IgY antibodies against bacterial infection

Development of candidate IgY antibodies against ESBL-producing gram-negative bacteria for oral therapy

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What matters is not the facts but

how you discover and think about them

Richard Dawkins

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List of abbreviations

amp- Ampicillin

ap1IgY- Affinity purified immunoglobulin Y against peptide 1

ap2IgY- Affinity purified immunoglobulin Y against peptide 2

AQP1- Aquaporin 1

aTIgY- Affinity purified immunoglobulin Y against TEM-1

BSA- Bovine Serum Albumin

CC- Cell culture

CDC- Centers for Disease Control

CFU- colony forming unit

CL-Cell lysate

DAPI- 4',6-diamidino-2-phenylidole

DMSO- Dimethyl sulfoxide

E-beam- Electron beam

EC- Enzyme Commission

ECDC- European Centre for Disease Prevention and Control

EEA- European Economic Area

eIgY- Affinity purified immunoglobulin Y against e-beam-inactivated *E.coli*

ELISA- Enzyme-linked immunosorbent assay

EMEA- European Medicines Agency

ESBL- Extended Spectrum Beta-Lactamase

Hch- Heavy chain

hIgY- Affinity purified immunoglobulin Y against heat-inactivated *E.coli*

HPLC- High-performance liquid chromatography

IgA- Immunoglobulin A

IgG- Immunoglobulin G

IgM- Immunoglobulin M

IgY- Immunoglobulin Y

KLH- Keyhole limpet hemocyanin

K_m- Michaelis constant

LB- Luria-Bertani medium

Lch- Light chain

MRSA- Methicillin-resistant *Staphylococcus aureus*

MS- Mass spectrometry

NMR- Nuclear magnetic resonance

OD- Optical density

p1- Peptide 1

p1IgY- Immunoglobulin Y against peptide 1

p2- Peptide 2

p2IgY- Immunoglobulin Y against peptide 1

PBS- Phosphate-buffered saline

PDB-Protein Data Bank

pI- Isoelectric point

rIgY- Random Immunoglobulin Y (non-specific)

RT- Room temperature

SDS-PAGE- Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SD- Standard deviation

TEM- ß-lactamase isolated from patient named Temoriera

TIgY- Immunoglobulin Y against TEM-1

VRE- vancomycin-Resistant *Enterococcus*

WHO- World Health Organization

Introduction

1. Introduction

1.1. Antibiotics in agriculture and human diseases

Antibiotics were first defined by Waksman more than 70 years ago as bacteriostatic, bactericidal and antifungal chemicals naturally synthesized by microorganisms. [1] Nowadays, the definition includes substances not only originated from natural products, but also those produced synthetically. [2] In 1946, the New York Academy of Sciences' conference was focused on pharmacological properties and clinical applications of two 'wonder drugs': Alexander Fleming's penicillin and Selman Waksman's streptomycin. Their discovery revolutionized the scientific world and opened the antibiotic era in human and veterinary medicine giving new opportunities in the battle against pathogenic microbes. [2, 3]

Antibiotics also play a very important role in agriculture and are widely used in livestock for growth control and promotion, disease prevention and treatment. [4, 5] Even though, the use of antibiotic growth promoters is banned in the European Union and has been relatively minimalized in the United States, they are still used extensively in other regions of the world. [6, 7]

The ubiquity of antibiotics is the result of the worldwide increasing need for meat. According to statistics, the USA is the largest meat consumer over the past thirty years with more than 100 kg meat per capita, followed by Brazil, EU, Russia and China. During the last five decades in the US, the average number of chickens and cattle on the farms doubled and the number of pork from the animal producers more than twenty-two fold. Till now the biggest meat producer is China, with the amount of beef, pork and chicken almost double over the European production (2013), and at the same time it was estimated that half of the antibiotic consumption (150,000-200,000 metric tons per year) goes to livestock. In 2012, 10,000 metric tons of antibiotics in the US and 8,000 in Europe were used in food animals. [8]

There is a broad spectrum of antibiotics used in livestock production: tetracyclines, amphenicols, penicillins, cephalosporins, first-, second-, third- and fourth-generation cephalosporins, sulfonamides, trimethoprim, macrolides, lincosamides, aminoglycosides, quinolones, polymyxins, pleuromutilins and ionophores. [4, 5] Many of them are also used in

human medicine. [9] Moreover, up to 90% of antibiotics are already prescribed in a primary care, even against common, non-serious infections such as e.g. sore throat. [10, 11] According to statistics, 30% of antibiotic therapies are prescribed to patients inappropriately. [12] Consequently, the increasing usage of antimicrobials leads to the resistance of bacteria which has become a major threat to public health in the XXI century.

1.2. Worldwide problem of antibiotic-resistance bacteria

The resistance to critically important antibiotics for human medicine can have unpredictable impact on health care and clinical procedures. Antibiotic resistance has been associated with more frequent and longer hospitalization, longer illness, a higher risk of invasive infection and a twofold increase in the risk of death. [13] The main problem comprises drug resistant foodborne pathogens such as *E.coli*, *Salmonella*, *Campylobacter*, *Staphylococcus* (including MRSA - methicillin-resistant *S.aureus*), *Enterococcus* (including VRE - Vancomycin-*Resistant Enterococcus*), *C.difficile*, *P.aeruginosa*, *Acinetobacter*, *Shigella*, and respiratory pathogens *S.pneumoniae*, *M.tuberculosis* and *K.pneumonia*. [14–16]

According to the Centers for Disease Control and Prevention (CDC) report from 2013 the annual number of infections in US caused by resistant *E. coli* and *Klebsiella* was 35,300 altogether, with 2,310 cases of death, for drug resistant *Salmonella*: 103,800 infected patients and ~40 deaths, for *Campylobacter*: 310,000 infections and 28 deaths, for *MRSA*: 80,000 infections and 11,000 deaths; for *VRE* 20,000 infections, 1,300 deaths, for *C.difficile* 250,000 infections and 14,000 deaths, for *P.aeruginosa*: 6,700 infections and 400 deaths, for *Acinetobacter* 7,300 infections and 500 deaths, for *Shigella* 27,000 infections and >5 deaths, for *Streptococcus* (with data regarding *S.pneumoniae*) 1,208,900 infections and 7,600 deaths; for drug resistant tuberculosis 1,042 infections and 50 deaths. [14] In Europe the situation does not look better.

According to the European Centre for Disease Prevention and Control/European Medicines Agency (ECDC/EMEA) Joint report from 2009 at least 25,000 people die every year in Europe due to antibiotic-resistant infections. The estimated number of annual infections of resistant *S.aureus* (*MRSA*) was 171,200 with 5,400 deaths, for *VRE* 18,100 infections and 1,500 deaths, for resistant *S.pneumoniae* 3,500 infections (data on deaths not published), for

resistant *E.coli* 32,500 infections and 5,100 deaths; for resistant K.*pneumoniae* 18,900 infections and 2,900 deaths, for resistant *P.aeruginosa* 141,900 infections and 10,200 deaths. [17, 18] The annual infection and death rates coming from resistant bacteria are summarized in Table 1.

Table.1. Annual numbers of infections and deaths caused by resistant strains of bacteria in Europe and USA, reported by European Centre for Disease Prevention and Control (ECDC) in 2009 and Centers for Disease Control and Prevention (CDC) in 2013. [14, 17, 18]

	Antibiotic-resistant bacteria	Annual number of infections	Annual number of deaths
CDC report USA 2013	*E.coli + Klebsiella*	35300	2310
	Salmonella	103800	40
	Campylobacter	310000	28
	MRSA	80000	11000
	VRE	20000	1300
	C.difficle	250000	14000
	P.aeruginosa	6700	400
	Acinetobacter	7300	500
	Shigella	27000	5
	Streptococcus	1208900	7600
ECDC report EU 2007	*E.coli*	32500	5100
	K.pneumoniae	18900	2900
	MRSA	171200	5400
	VRE	18100	1500
	S.pneumoniae	3500	Data not published
	P.aeruginosa	141900	10200

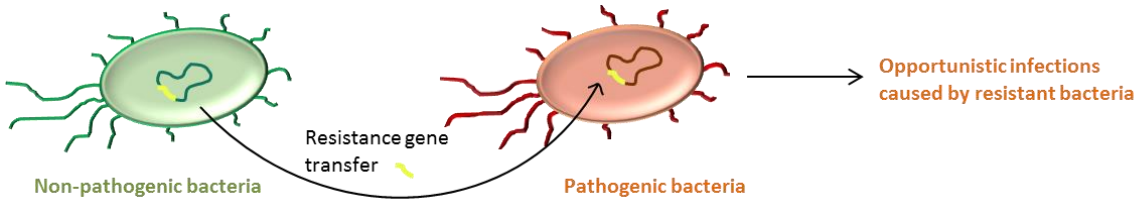
Another report of ECDC on antimicrobial resistance of *Salmonella*, *Campylobacter*, *E.coli* and *S.aureus* isolates from humans, animals and food published in 2013 brings bothering analysis. [19] 36.1% of *Salmonella* isolates from human were resistant to ampicillin, 35.7% to sulfonamides and 34.5% to tetracyclines, whereas 31.8% showed multi-resistance. *Salmonella* isolated from meat showed resistance to tetracyclines, ampicillin and sulfonamides in the

range from moderate to extremely high. Furthermore, 56% of *Salmonella* isolates from broilers were multi-resistant, as well as 37.9% from pigs and 73% from turkeys. *Campylobacter* samples from patients showed very high resistance to the clinically important antibiotics: more than 50% of all isolates were resistant to ciproflaxin, 33.5% isolates of *C.jejuni* and 58.1% of *C.coli* were resistant to tetracycline, 13.4% of *C.coli* isolates were resistant to erythromycin. *C. jejuni* and *C. coli* isolates from avian showed from high to extremely high resistance to ciproflaxin, nalidixic acid and tetracyclines, comparable to those from pigs and cattle. Percentage of resistant *E.coli* isolates from broilers and pigs were in the range of 6.3%-64.7% and 17.7%-88.0% respectively. [19] *E.coli* isolated from avian, pigs and cattle showed resistance in the range of moderate to very high to: ampicillin (13%-58.6% of isolates), sulfonamides (20.2%-48.6% of isolates), tetracycline (23.2%-52.8% of isolates), streptomycin (17.6%-50.4% of isolates) and nalidixic acid (55.4% of isolates, data referred only to broilers). *E.*isolates (*E. faecium* and *E. faecalis*) showed very high resistance to tetracyclines (from broilers 61.6%-87.0% of isolates, from pigs 45.6%-85.5% of isolates, from cattle 30.8%-85.5% of isolates) and erythromycin (from broilers 59.0%-60.6% of isolates). The resistance of *Enterococcus* to quinupristin/dalfopristin was extremely high among all types of tested animals, giving the number of 73.7%-94.7% of investigated isolates. Analysis of *S.aureus* in meat from turkeys, pigs, broilers and cattle was narrowed down to *MRSA,* and the number of its infection increased from 2.2% in 2009 to 20.8% in 2013. [19] The ECDC Annual epidemiological report from 2014 which shows that *MRSA* is above 25% of isolates in 7 of 29 analyzed countries in EU/EEA. The percentage of aminoglycoside-resistant *E.faecalis* isolates is between 25%-50% among reporting countries. More than 50% of *Acinetobacter spp.* isolates were resistant to carbapenems, fluoroquinolones and aminoglycosides. Above the 10% of *P.aeruginosa* isolates in 19 of 29 reporting countries were resistant to carbapenems, and 14% showed multi-resistance. Resistant to both penicillin and macrolides was 10% of *S.pneumoniae* isolates within 10 from 28 reporting countries. Moreover, due to data from 2012 in EU/EEA countries 11.9% of *E.coli* isolates were resistant to third-generation cephalosporines and 4.4% showed multi-resistance. For *K.pneumonia* the percentage of isolates resistant to third-generation cephalosporines was 25.6% and 18.2% was multi-resistant. [18, 20]

1.3. From harmless bacteria to the spreading of dangerous pathogen

Scientist, medical doctors, authorities from health organizations, all together agree that the resistance is a consequence of ubiquity of antibiotics in animal farming, industry and therefore in environment, but also because of overuse and misuse of antibiotics in human and veterinary medicine. [11, 13, 15, 21–23] Bacteria, which are known for their adaptive capabilities, have developed several mechanisms to fight against antimicrobial drugs. Even shortly after first uses of ampicillin in the middle of the XX century, it was noticed that bacteria are able to destroy its activity and survive. [3] Antibiotic resistance has its origins in gene mutations or in genetic elements exchange (such as plasmids, transposons, gene cassettes, integrons) between bacteria strains through the principle of horizontal transfer. It includes three mechanisms: transformation (uptake of naked DNA), conjugation (direct cell-to-cell transfer of genes), and transduction (bacteriophage plays the role of a DNA vector). [18, 24, 25] Fast and direct sharing with resistant strategies is evolutionary-wise and gives bacteria an unquestionable advantage in the antibiotic battle. It is especially dangerous when an already pathogenic bacteria strain gains new resistance genes, and as a consequence can lead to the development of opportunistic infections not responding to current treatment (Fig.1).

Fig.1. Exchange of antibiotic resistance genes between bacteria.



Additionally, interactions between bacterial cells can lead to the development of a biofilm community, which structure increases the resistance to environmental stress influenced by antimicrobial agents. The antibiotic resistance of bacteria in a biofilm can be 1000 times higher than in planktonic cells, what enhances the risk of failure of antibiotic treatment even more. [26] Resistance can be spread easily in many different routes, linking human population with agriculture. The net of correlations is shown in Fig.2.

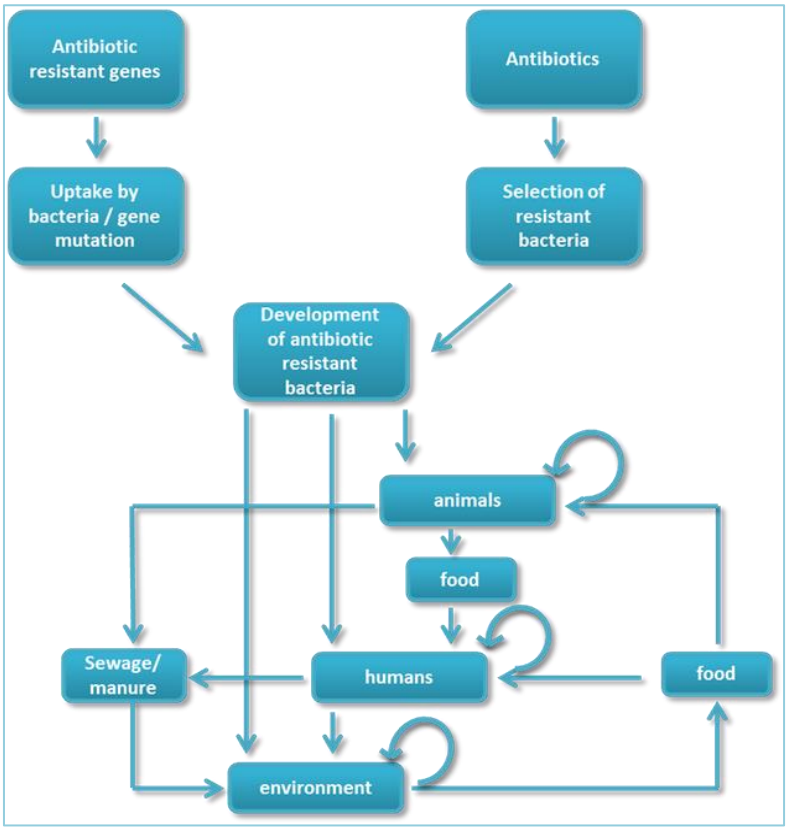


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Fig.2. Scheme describing the spread of antibiotic resistance. [18] Antibiotic resistance can be the result of two processes: 1) genetic changes of bacteria caused by uptake of gene elements or mutations, 2) natural selection after contact with antibiotics. Resistance can be easily spread bewteen human, environment, agriculture and livestock.

Animals, humans, agriculture products and environment (soil) are possible sources of resistant pathogens. Bacteria can be transferred between all of them through contaminated vegetables, meat, sewage, manure or by direct contact with infected animals or people. [15, 25, 27, 28] Therefore, farms and hospitals should be considered as places of increased risk. Due to the effect of 'global village' and rising number of travelers, the epidemiology of antibiotic-resistance can get out of control by dissemination across the borders. [18, 29, 30]

1.4. Mechanism of antibiotic resistance

Different molecular mechanisms of resistance are described: antibiotic target modification, compound chemical modification, active efflux or molecular bypass.[31] The first option is based either on genetic point mutations (e.g. mutation in DNA gyrase leads to resistance to synthetic fluoroquinolone antibiotics - ciprofloxacin) or efficient and selective enzymatic activity within microorganisms (e.g. in ribosome methyltransferases). The result in both cases is a special conformation change in the bacterial target of the drug – as a consequence antibiotic cannot bind and loses efficiency of its molecular strategy of action. The same happens in so called molecular bypass. This is a process of replacement of antibiotic sensitive molecular targets by neutral ones, for example vancomycin resistance is a result of replacement of an amide with an ester. Another example is chemical modification, where antibiotics are inactivated by enzymatic catalysis (e.g. ß-lactamases destroy ß-lactams by hydrolyzing their four-atom ring). While the mechanism of efflux pump, supported by membrane proteins, helps bacteria actively remove antibiotics extracellularly. [18, 26, 31] Further chapters are focused on the strategy based on antibiotic-degrading enzymes – ß-lactamase mediated resistance. All the mechanisms described above are showed schematically on the Fig. 3.

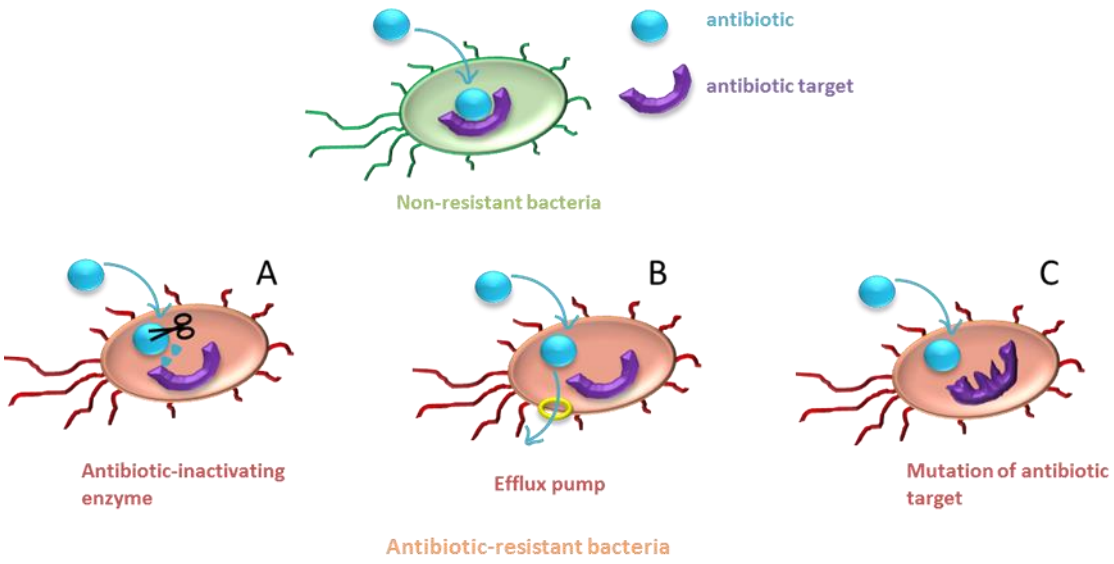


Fig.3. There are different molecular mechanisms involved in antibiotic resistance: a) inactivation of antibiotic by enzymatic catalysis, b) elimination of antibiotic by active efflux, c) molecular change of the target so antibiotic cannot bind.

1.5. Beta lactamases

While in 1940s the world seemed to have found a gold remedy against bacterial infections - penicillin, Abraham et al. soon reported mechanism of its inactivation by ß-lactamase. [32] Beta lactams are the biggest group of antibiotics, which mechanism of action blocks the synthesis of the bacterial cell wall. During the cell wall synthesis, single building blocks of peptidoglycan (murein) are crossed-linked by D-Ala-D-Ala carboxypeptidase. Four-atom ring of beta-lactam antibiotics perfectly mimic the structure of D-Ala-D-Ala residue and competitively inactivate the catalytic site of peptidase, so the peptidoglycan polymer cannot be formed. As a consequence bacteria undergo osmotic instability and die. Bacterial enzymes called ß-lactamases, hydrolase ß-lactam ring of antibiotics and destroy their antibacterial properties. In the 1960s, the first ß-lactamase was described – TEM-1 was synthesised by *E.coli* strain isolated from a Greek patient Temoniera, after whom the enzyme was named. [33] Shortly after, different ß-lactamases were detected in other family members of *Enterobacteriaceae*, as well as in *K.pneumoniae*, *P.aeruginosa* or *A.baumanii* worldwide, showing resistance activity against growing group of ß-lactam antibiotics. Until now 1,886 different ß-lactamases were identified. [34–37] ß-lactam resistance genes can be encoded on chromosomes or on plasmids, in both gram negative and gram positive bacteria. [38, 39] In gram positive bacteria, ß-lactamases are synthesised extracellularly, in gram negative cumulate in the periplasmic space. There are two schemes of ß-lactamase classification and they are based on differences in: functionality – Bush-Jacoby-Medeiros, and structure (protein sequence) – Ambler. [35, 36, 39] Even though functional classification provides selective ß-lactams resistance profiles, the structural scheme is easier to analyse and less controversial, considering such a huge diverse group of enzymes. Based on differences in amino acids sequences, Ambler proposed 4 classes of ß-lactamases: A,B,C and D. [36, 39, 40] A diagram of the sequence similarity network between ß-lactamases variants is shown on Fig. 4. [37] In the classes A,C and D the mechanism of ß-lactam ring hydrolysis is active Serine-based, class B is a group of metallo-ß-lactamases which require a Zink ion for this reaction. Classes A, C and D share a similar motif of amino acids including presence of Serine and Lysine residues in the active site, which is situated between two domains α and α/ß. Examples of ß-lactamases from classes A, C and D are depicted in Fig.5. [40]

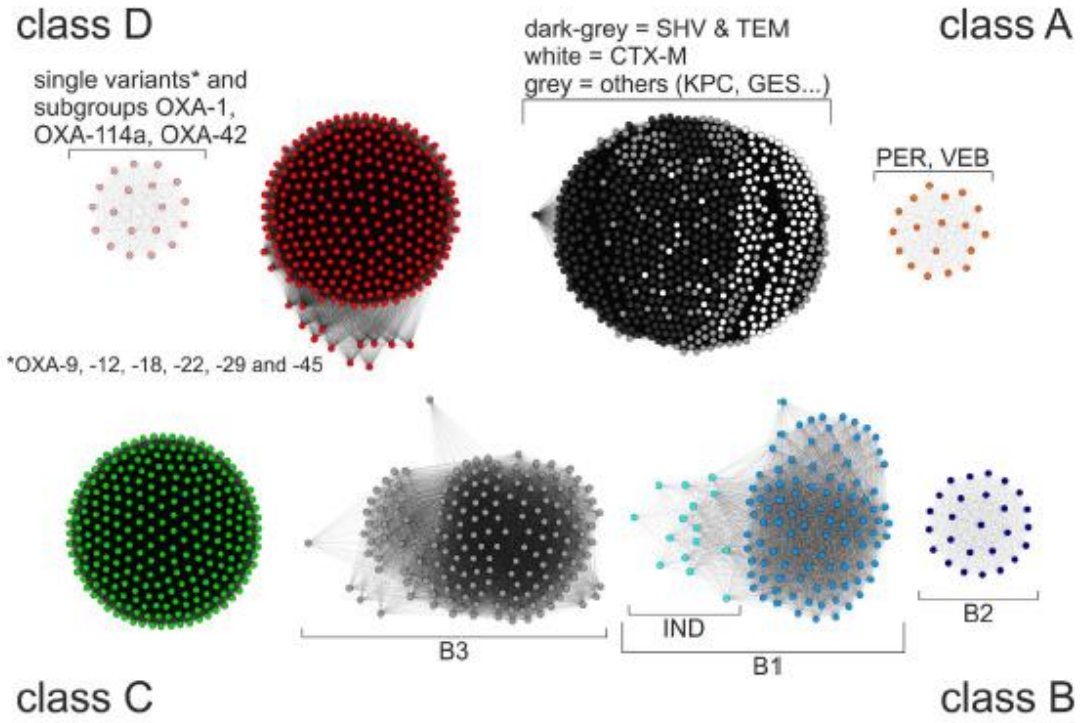


Fig.4. ß-lactamase sequence similarity network based on 1,494 variants. Behind each dot stands a unique amino acid sequence.[37]

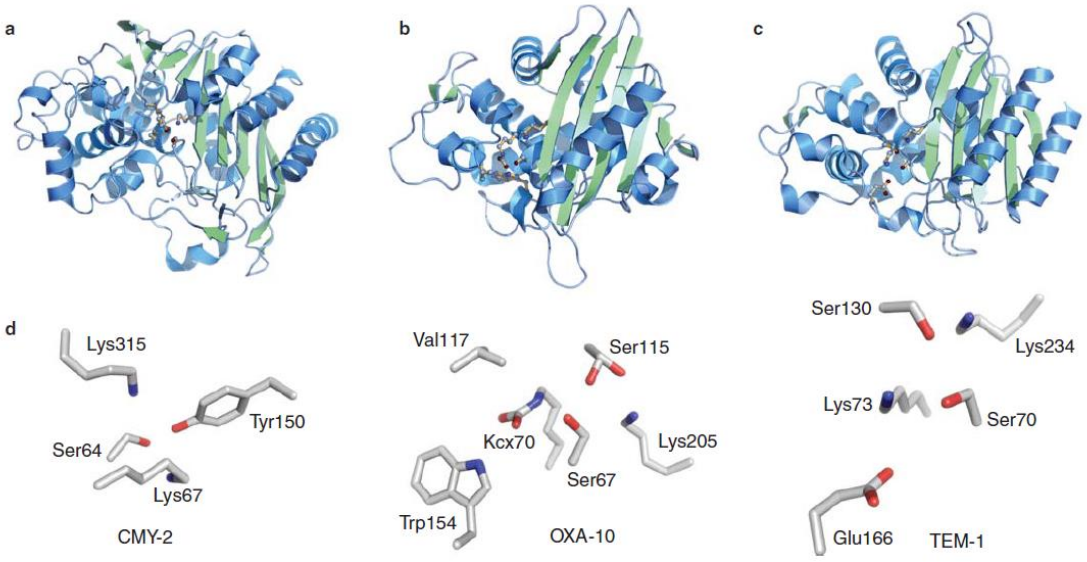


Fig.5. Structure and function of A, C and D class ß-lactamases show many similarities. Three representatives are presented: a) class A- TEM-1, b) class C- CMY-2, c) class D- OXA-10. ß-sheets are green-, α-helixes blue- and loops gray-coloured. In all these classes the active site contains Serine and Lysine residue necessary for the mechanism of hydrolysis of the ß-lactam amid bond (d). [40]

Moreover, the sequence and 3D structure homology suggest that class A and C might have evolved from the same origin. [41–45] Most of ß-lactamases which became clinically relevant are those plasmid-encoded, synthesised in gram negative bacteria and mainly incorporated into class A. Molecular class A (functional group 2) is also the one which grew the most last years with the highest number of newly reported naturally occurred enzymes (Fig. 6). [46]

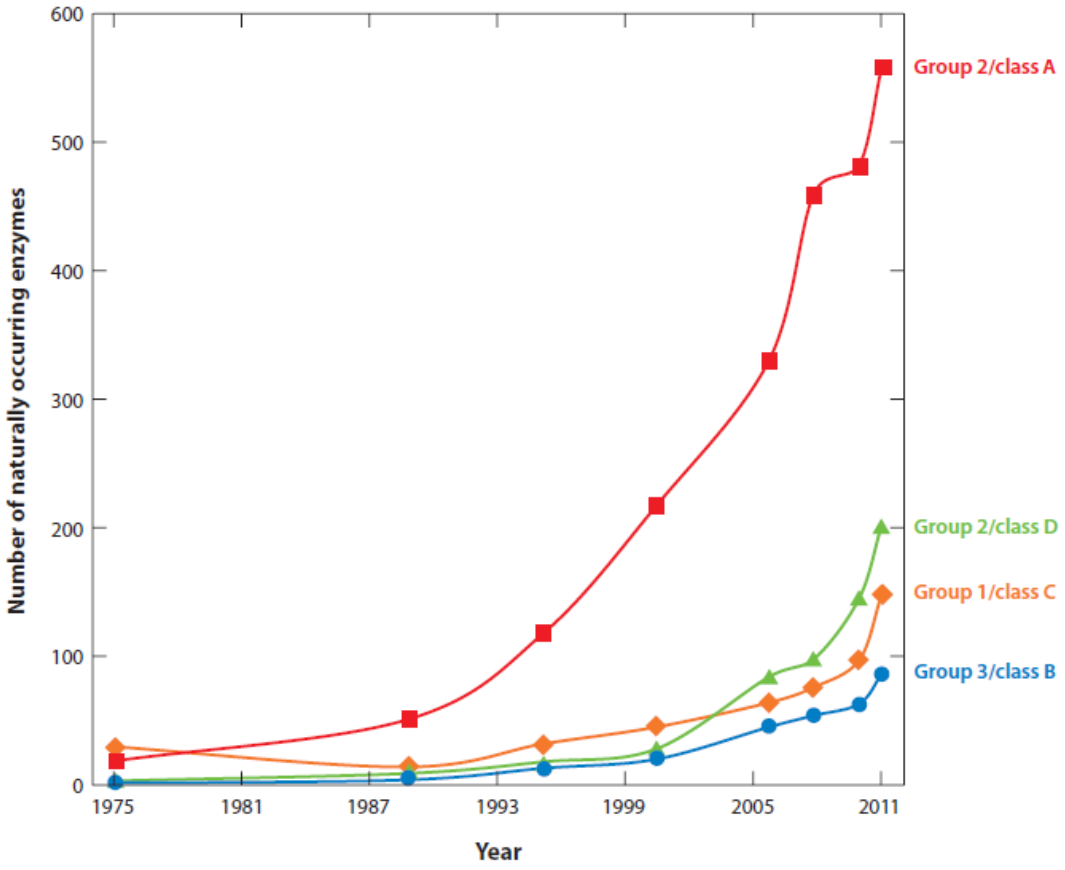


Fig.6. Number of newly reported naturally occurring ß-lactamases from different classes (functional/molecular) over the years. Among all the classified ß-lactamases, the group 2/class A was the one which expanded the most. Figure adapted from American Society for Microbiology, 2010 by Bush et al. [46]

1.6. Class A ß-lactamases

Class A is a group of broad spectrum ß-lactamases hydrolyzing penicillins, cephalosporines, in some cases monobactams and carbapenems. [35] This group is mostly represented by TEM and SHV types, with parental enzymes TEM-1 and SHV-1 sharing 68%

sequence homology. Another fast growing type is CTX-M but it stands phylogenetically further from TEM and SHV. [46, 47] This class is clinically important as it is a source of Extended Spectrum ß-lactamase mutants (shown in Fig. 7). [37]

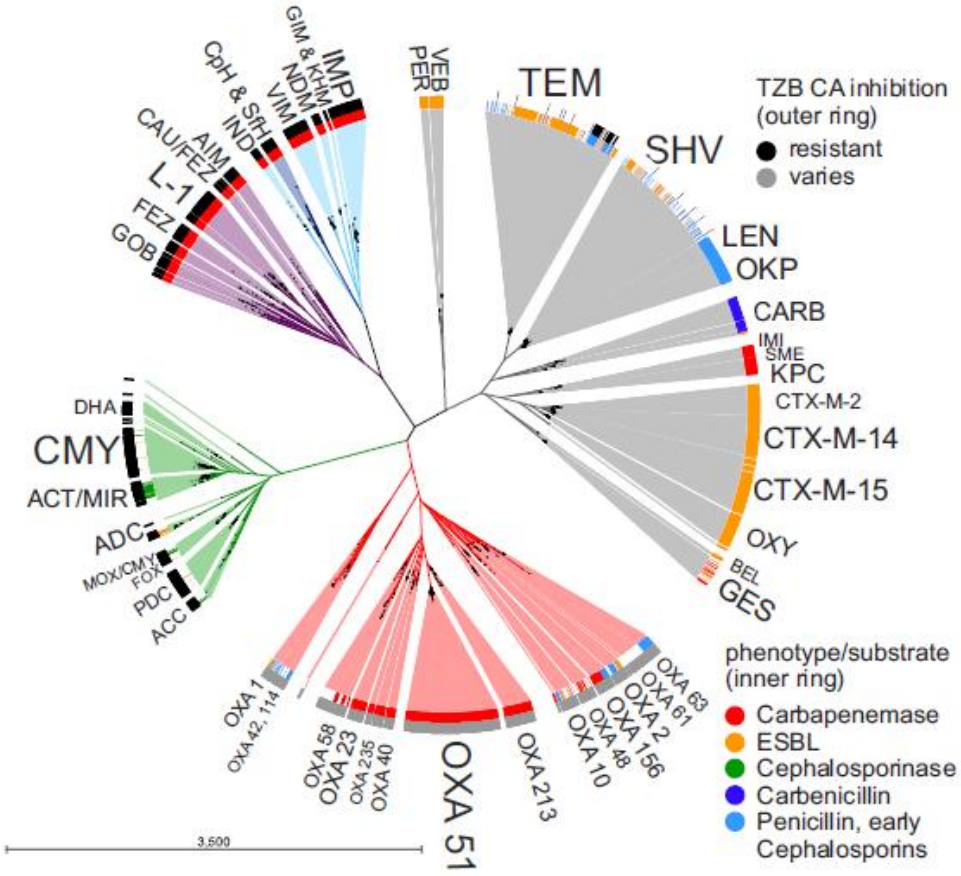


Fig.7.Phylogenetic tree of 1,886 ß-lactamases. Different Ambler's classes are colored in: grey- A, light blue- B1, dark blue- B2, purple-B3, green- C, D-red. CA stands for Clavulanic acid, TZB- tazobactam. Diagram indicates that class A is the source of ESBLs.[37]

Based on crystallographic structure analysis of these enzymes, two domains can be distinguished: α (with 8 α-helixes) and α/ß (with 3 α-helixes and 5 ß-strands). [42, 48] The active site is placed between these two domains, and contains highly conserved residues: Ser70, Ser130, Lys73, and Lys234 which take part in the process of ß-lactams hydrolysis. [49, 50] The important structure is the Ω-loop which is placed between amino acid number 161 and 179 and hangs above the active pocket. It contains another two conserved residues Glu

166 and Asn 170 important for catalytic function of the enzyme. [40, 47, 50, 51] The active site based on crystal structure of TEM-1 and SHV-1 can be viewed in Fig. 8.

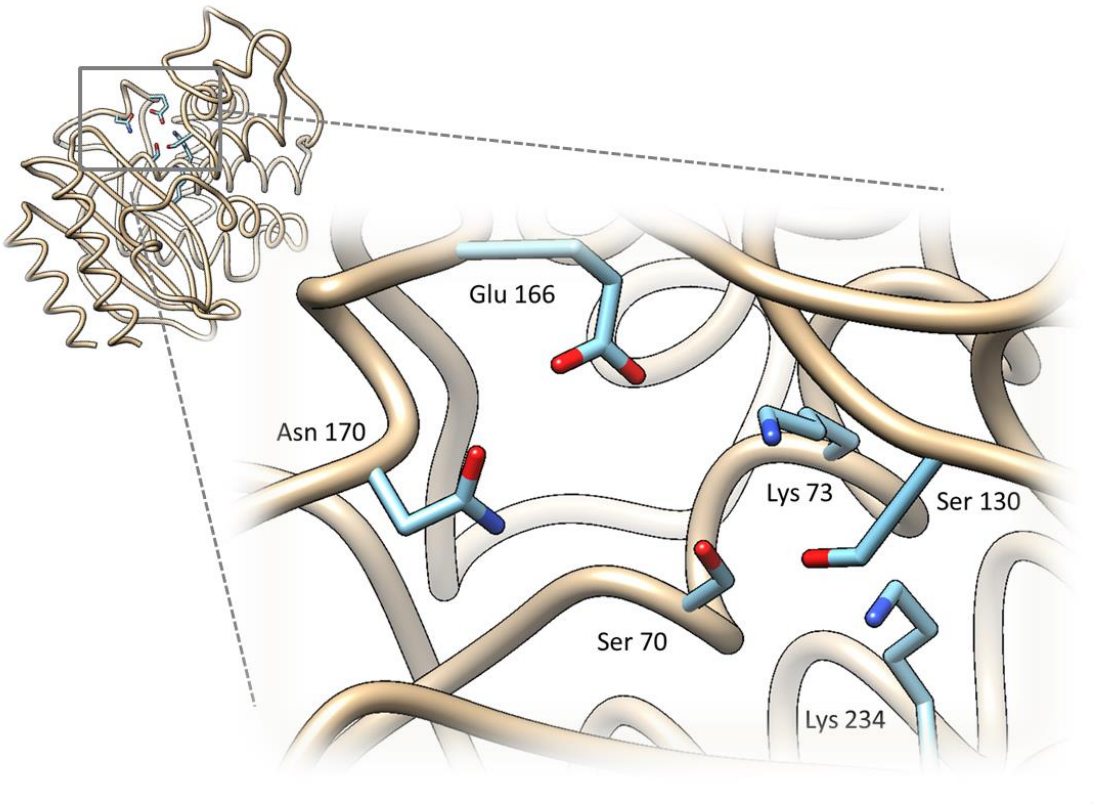


Fig.8. Conservative residues in the active site of A class ß-lactamases. Asn170 and Glu166 are situated on the Ω-loop which creates a rim over active pocket with residues: Ser70, Lys73, Ser130 and Lys 234. Structure was created with the Chimera program, based on crystal structures of TEM-1 and SHV-1 (Pdb: 1axb and 1shv). [52]

For the whole A-class of ß-lactamases the reaction of hydrolysis of ß-lactams is a cycle process based on two processes: acylation and deacylation, where the main role is played by Ser70 attacking the ß-lactam carbonyl of the antibiotic. [53, 54] Acylation is the reaction of creating an acyl group on another chemical compound through the reaction with a hydroxyl group. In this situation the hydroxyl group carrier is amino acid residue Ser70 from ß-lactamase. A non-covalent enzyme-substrate complex (Henri-Michaelis complex) formation is the first step of the acylation reaction (Fig. 9). [55] A nucleophilic attack on the ß-lactam

carbonyl by the hydroxyl group of Serine is catalyzed by the presence of a general base (this can be either Glu166 or Lys73). [56, 57] Two mechanisms are likely possible: a proton is transferred to Lys73 directly or via hydrolytic water positioned and coordinated by Glu166, Asn170 and Ser70. [50] In the next step, an oxygen from Ser70 residue is ready to attack a carbonyl carbon of the ß-lactam ring, what proceeds through the formation of a tetrahedral intermediate. It is also proposed that meanwhile the Lys73, Ser130 and Lys234 residues take part in the protonation of a nitrogen from the ß-lactam ring. [57–61] Consequently, an amid bond breaks and a acyl-enzyme adduct is formed. Next, the hydrolytic water molecule is activated by a general base Glu166, attacks the acyl-enzyme adduct and as a result a tetrahedral intermediate is formed. [58, 62] Lys234 is proposed as a stabilizing residue for this structure. [55] Then, as a consequence of deacylation, the ß-lactam ring of antibiotic is hydrolyzed and the tetrahedral intermediate turns into an enzyme-product complex. Beta lactam loses its primary activity due to the conformation changes. [42, 58] The scheme of acylation and deacylation process is shown on Fig. 10.

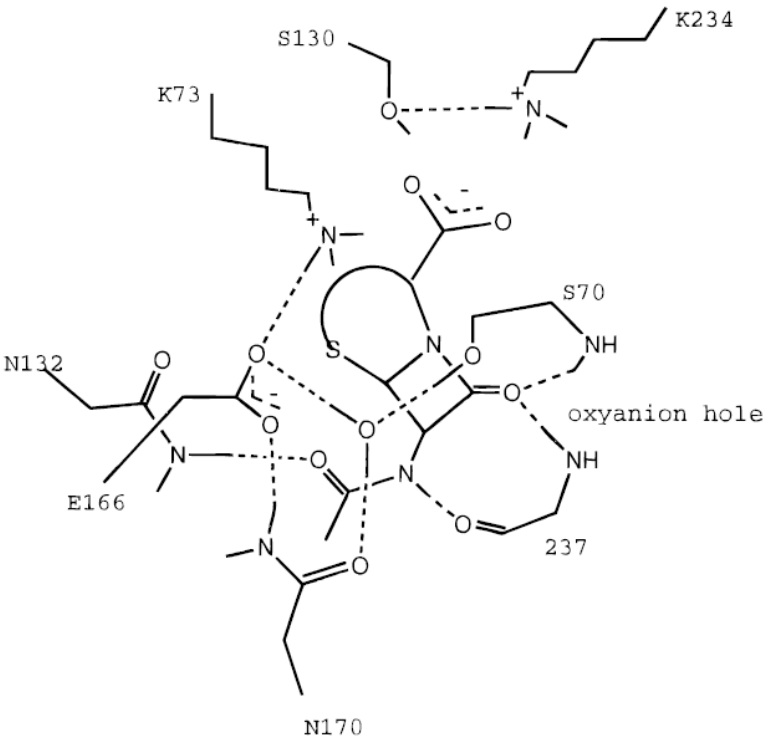


Fig. 9. Henri-Michaelis complex of ß-lactam antibiotic and A-class ß-lactamase.[55]

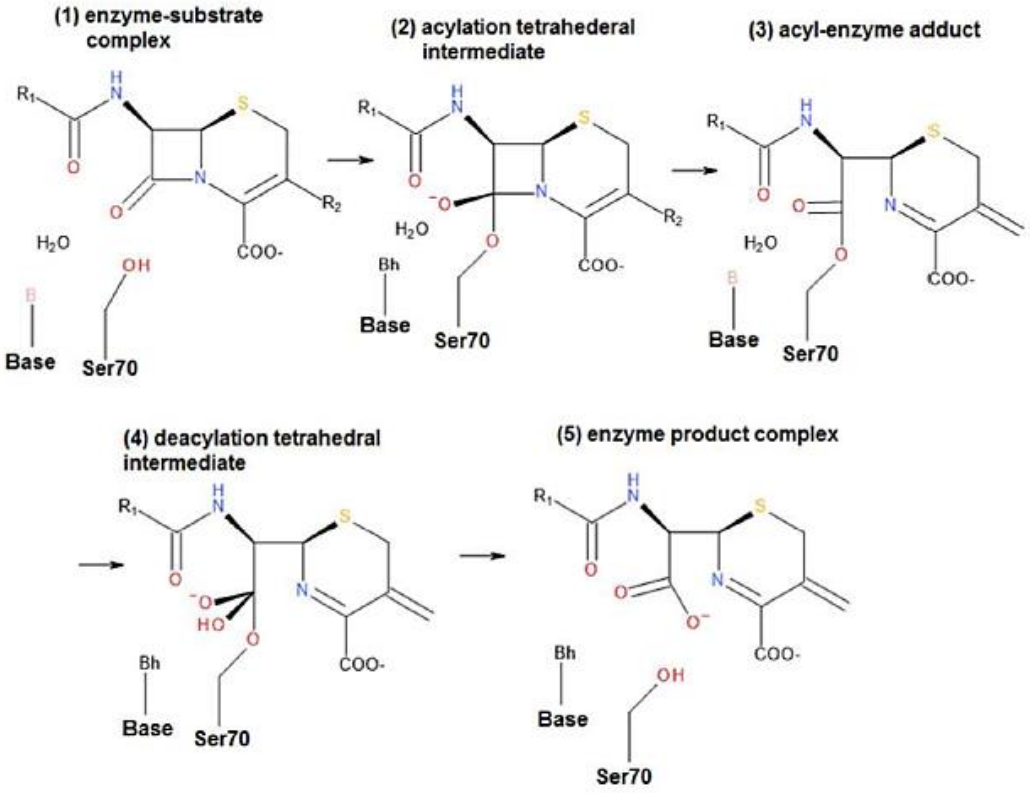


Fig.10. The hydrolysis of ß-lactams by A class ß-lactamases is a cyclic process based on two processes: acylation and deacylation. After the formation of a non-covalent enzyme-substrate complex, in the presence of a general basis, Ser70 residue attacks the amid bond of ß-lactam. Through the acylation reaction an acyl-enzyme adduct is formed. Due to the hydrolytic water activation, the acyl-enzyme adduct undergoes a deacylation reaction and turns into an enzyme-product complex. As a result, the amid bond of ß-lactam is hydrolyzed and loses its functionality, and ß-lactamase is regenerated ready for another reaction cycle. [57]

1.7. TEM-1 and ESBLs

TEM-1 is a plasmid encoded, 29 kDa atomic mass enzyme, representing the predominant group of TEM type ß-lactamases providing antibiotic resistance within the *Enterobacteriaceae* family. Its hydrolytic profile indicates activity against penicillins, first generation cephalosporins – cephaloridine, cefazolin, second generation cephalosporins - cephalotin, but not against the third generation of cephalosporins (cefotaxime, ceftazidime) and monobactam aztreonam, and it is inhibited by clavulanic acid (CLA) and tazobactam (TZB). [46, 63] Like it is mentioned in chapter above, TEM-1 as a member of class A enzymes, has two domains α and α/β, with the active site placed between them. Two of the most important and conserved catalytic residues Lys73 and Ser70 are located on the helix H2. Together with Glu166 and

catalytic water molecule participate in acylation step in reaction of the ß-lactam hydrolysis. [59, 64]

Other important non-mutable residues are situated in a Ω-loop: Glu166 and Asn170. Asn170 helps in positioning the catalytic water molecule by forming the hydrogen bond through the side chain of the residue. Then, it enables the Glu166 to activate the water molecule in the deacylation step. [42, 58, 65–68] A visualization of ß-lactamase TEM-1 can be found in Fig.11.

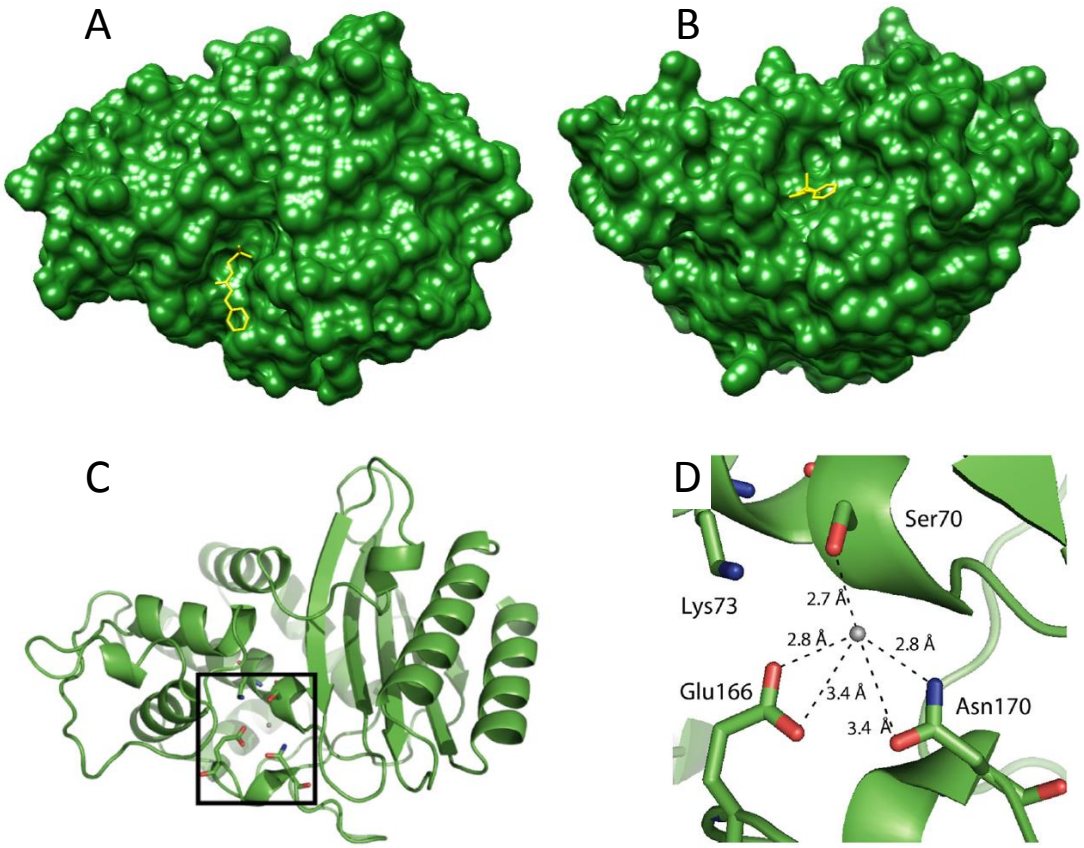


Fig.11. Visualization of ß-lactamase TEM-1. A, B - 3D Crystal structure of a phosphonate- TEM-1 complex visualized in the Chimera. Phosphonate belongs to the structure of inhibitor used in this crystallographic study. [52, 69] C – cartoon picture of TEM-1 with active site in the black square. D- conserved amino acid residues of active site including these responsible for positioning of the catalytic water, colored in grey.PDB 1BTL.[50]

The Ω-loop forms a rim over the active pocket of TEM-1 and it has a huge impact on the structure and function of the enzyme. It is directly involved in substrate interaction, water

coordination necessary for catalysis, and is mutation-sensitive. [58, 70, 71] B-lactamases are highly mutable enzymes, single substitutions of amino acids can change their interaction with a substrate, leading to the development of new mutants with an enlarged antibiotic hydrolysis profile - so called Extended Spectrum Beta Lactamases (ESBLs). Apart from possessing a standard ß-lactamase functionality, they are able to hydrolyze the third generation of cephalosporins and aztreonam. These TEM-type derivates have been developed as a consequence of mutations in *bla*$_{\text{TEM-1}}$ gene resulting in amino acids substitutions, which mainly occur within or near the active site, alone or combined and mostly appear as follow: Glu104→Lys, Arg164→Ser/His, Gly238→Ser, Glu240→Lys. [72, 73] Some examples of TEM-1 mutants with ESBL profile are shown in Fig. 12.

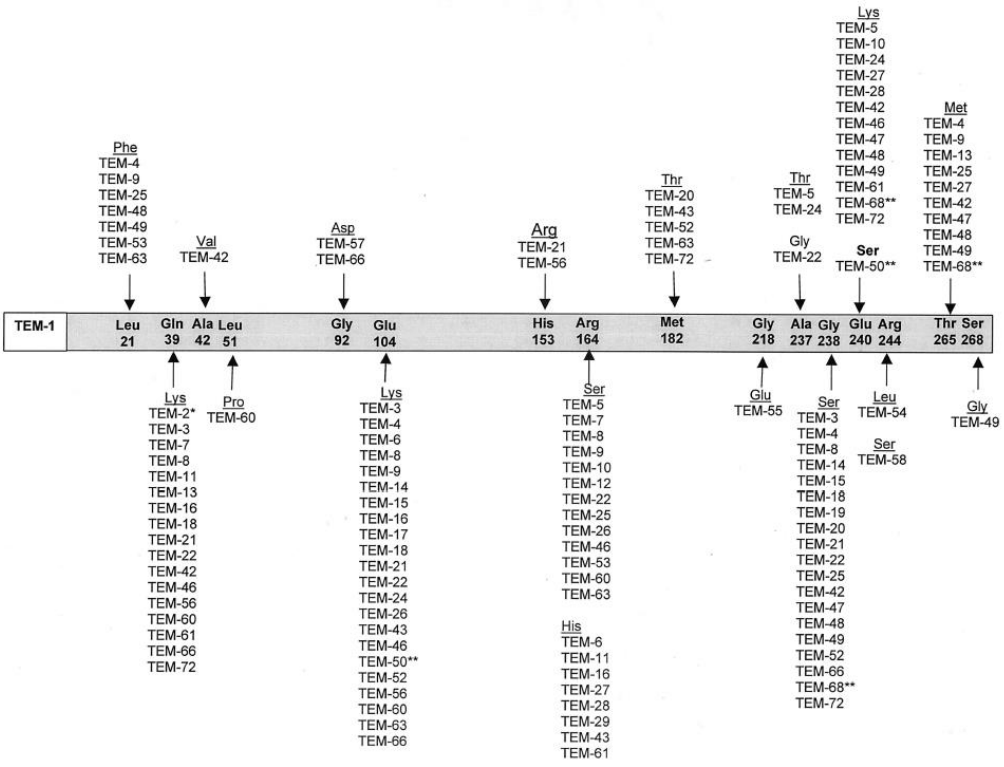


Fig.12. TEM-type ESBL derivatives. Examples of amino acid substitutions which provide the ESBL phenotype in newly developed TEM ß-lactamases in comparison to TEM-1 sequence marked in gray. TEM-2 does not have a ESBL hydrolysis profile but was included in the figure as a TEM-1 derivate. [73]

These amino acids mutations can change the flexibility of the Ω-loop, the size of a space around the active pocket where the substrate is bound or stabilize the conformation of the

enzyme. [63, 66] These changes enable ESBLs to hydrolyze new generations of antibiotics. As an example of a TEM-1 mutant can be a clinically found ESBL TEM-52, which is able to hydrolyze additionally the third generation cephalosporin (cefotaxime). Even though mutations did not cover the active site residues of the enzyme, the set of important functional substitutions E104K/G238S/M182T changed the conformation of the loops near the active site. [74] It resulted in the change of the pI value for TEM-52 and its kinetic parameters in the interaction with penicillins, first- and third- generation of cephalosporines. (shown in Tab. 2. and 3.)

Tab.2. Comparison of amino acid residues at critical positions and isoelecric point (pI) values between TEM-1 and TEM-52. [75]

ß-lactamase	**pI**	**aa substitution**			**Ref.**
		104	182	238	
TEM-1	5.4	Glu	Met	Gly	[76]
TEM-52	6.0	Lys	Thr	Ser	[75]

Tab.3. Comparison of Michaelis constant values (K_m) between TEM-1 and TEM-52. TEM-52 shows smaller values of K_m in reaction with penicillins and I-generation of cephalosporins, this means the affinity between substrate and enzyme is stronger than with TEM-1, and as a result the efficiency of antibiotic hydrolysis is higher. Moreover TEM-52 as a ESBL hydrolyses III-gen cephalosporins, while the K_m of TEM-1 ß-lactamase in reaction with this group of antibiotics could not be determined. [75]

	substrate	**K_m (µM)**	
		TEM-1	TEM-52
penicillins	Benzylpenicillin	26	5.3
	Ampicillin	40	4.1
I-gen. cephalosporines	Cefaloridine	244	13
III-gen cephalosporines	Ceftazidime	-	239
	Cefotaxime	-	30

There are two almost opposite hypotheses explaining how the E104K/G238S substitutions influence the TEM-52 ability to hydrolyze cefotaxime. It depends on the type of the study – crystallography or new drug design technique called Boltzmann docking. Importantly, in comparison to previous generations of cephalosporins, the third generation has a bulkier structure. The theory based on crystallographic models proposes that these amino acid substitutions open up the active site via motion of the loop between residues 238 and 242. It enables the enzyme to bind larger substrates and as a result to hydrolyze them. [77, 78] The hypothesis built on molecular dynamics simulations and Markov state models, which are the base of the Boltzmann docking approach, says that E104K/G238S substitutions do not change the conformation of the 238-loop but decrease the flexibility of the Ω-loop and pins it down towards the active pocket. This conformational change, together with the change in charge, promotes the heterogenicity in a substrate binding and enhances the hydrolysis rate. [74] Additionally, the mutation M182T stabilizes TEM-52 by suppressing aggregation and misfolding of protein caused by other substitutions. [79]

This mechanism shows how efficiently ß-lactamases can provide the resistance to newly introduced antibiotics through fast genetical evolution. The outbreak of ESBLs can be explained by selective pressure coming from the mass scale introduction of aminopenicillins, III-generation of cephalosporin and aztreonam. [46, 80] On the other hand, it can be supported by naturally occurred mutants, which exchange resistance encoded genes with clinical strains. Due to the fact that penicillins and cephalosporins have their origin in microbiological organisms, resistance against them could have been developed by bacteria in parallel. Nowadays more than 200 TEM type variants were identified and ~86 of them have ESBL phenotype. [36, 70, 81]

1.8. ESBLs in negative gram bacteria

In 2017, the WHO published a report entitled “The global priority list of antibiotic resistance bacteria to guide research, discovery and development of new antibiotics” grouping pathogens according to the level of their resistance: critical, high and medium. [82] The critical group encompasses extended spectrum beta lactamase producing *Enterobacteriaceae*. ESBLs are strongly related to *Gama Proteobacteria* class, especially to *Enterobacteriaceae* family including species such a *E.coli* and *Klebsiella*. [37] These gram negative bacteria differ from

gram positive species in cell wall structure morphology. The cell wall of gram negative bacteria consists of a thin layer of peptidoglycan polymers outstretched in the periplasmic space, which is closed by a cell membrane and an outer membrane. The outer membrane contains in its structure porins transporting sugars, ions and amino acids, efflux pumps moving toxic substances and antibiotics out of cells, and lipopolysaccharides which determine endotoxin properties. [83] Among gram negative bacteria, the mostly identified types of ESBLs are TEM/SHV or CTX-M, which are synthesized constitutively and secreted in the periplasmic space. These enzymes are reported worldwide in clinical isolates. [36, 84–93]

Enterobacteriaceae can be common human commensals. Although, *E.coli* and *Klebsiella* are present in healthy individuals e.g. in gastrointestinal tract or genitourinary tract, pathogenic strains, especially these producing ESBLs, can cause serious clinical diseases like intra-abdominal abscess, cholangitis, gastroenteritis, peritonitis or infections of urinary tract and severe diarrhea (mostly in developing countries). [91, 94–96]

Moreover, the gastrointestinal tract is considered as a reservoir of resistant *Enterobacteriaceae* and as the environment which favors the transmission of antibiotic resistant genes between microorganisms. [97–101] Additionally, the uses of new generation antibiotics e.g. cephalosporines can also, as a side effect, support the growth of new ESBL mutants. By eliminating a treatment-sensitive bacteria (not only pathogens but also good flora), the gastrointestinal environment becomes less nutrition-competent, hence, favors the colonization of new ESBL producing *Enterobacteriaceae*. This phenomenon is called the selective pressure and it is one of the explanations why it is so difficult to find a long-term effective antibiotic. [97] This is a serious threat for patients suffering from diseases that diminish the organism's condition and immune system, and require long hospitalization. [91, 102–104]

There are several guidelines for the antibiotic treatment against ESBL-associated bacterial infections, although the options get narrowed down fast due to the limitations of the pharma industry and the speed of new resistant strains development. [105–107] ESBLs are already resistant to almost all ß-lactam antibiotics, with exception to cephamycins and some carbapenems. Additionally, plasmids encoding ESBLs, very often also contain other antibiotic resistant genes. Moreover, the medical history of a patient has the biggest influence on the effectivity of chosen therapy: past treatments with antibiotics, implants or prosthetic devices, and also metabolic diseases. [108, 109]

Recommended antibiotics against gram negative ESBL-producing bacteria include: colistin, fosfomycin and temocillin. An alternative option is the bacteriostatic drug – tigecycline. There are also strategies combining two compounds, such as: a dual-carbapenem therapy, a new generation cephalosporine + ß-lactam inhibitor (e.g. Ceftolozane/tazobactam) or a III-generation cephalosporin + non-ß-lactam inhibitor (e.g. Ceftazidime/avibactam). [106] Unfortunately, all of these treatments are not always the final remedy for infection and additionally, can cause side effects such as nausea, diarrhea, headache, and fever or promote further resistance development. That is why scientist place great hopes on prophylactics and alternative treatment strategies. One of these is a strategy based on antibodies, using its natural potential of binding and inactivating toxic substances and pathogenic microorganisms.

1.9. Concept of passive immunotherapeutics

Passive immunization is a concept of administering specific antibodies obtained from a donor or developed commercially, to a patient or animal, as a prophylaxis or therapy. Naturally occurring examples of this process are: transfer of immunity from a mother to a child in milk and transfer of IgY antibodies from a hen to egg yolk of offspring, in mammals and avian, respectively. The first use of passive immune properties, dates back to end of the XIX century, when Emil von Behring treated diphtheria and tetanus with rabbit serum therapy. He received the Nobel Prize in Physiology and Medicine for this in 1901. [110] Next decades serum therapy was clinically used against pneumococcal pneumonia, scarlet fever and meningitis until the newly discovered antibiotics replaced them. [111] The interest in passive therapy has returned though, with the growing problem of antibiotic-resistant bacteria. It mainly refers to the introduction of specific mammal-origin Immunoglobulin G, what can provide short-termed but straightaway protection or neutralize pathogenic units (e.g. bacteria, viruses, toxins). There are already some licensed medical products based on polyclonal antibodies against infectious diseases such as: *C.botulinum* toxin, Cytomegalovirus, Hepatitis A and B, Rabies, Measles, Tetanus, Vaccinia, Varicella Zoster. [112] Also, due to hybridoma technique of producing monoclonal antibodies, passive immune therapies has started to develop in the field of cancer, immune diseases and allergy. [112–114]

Unfortunately, the limits of this approach are still: high costs, small quantities of antibodies and ethical concerns regarding welfare of animals. In the last two decades, a great interest in IgY technology has been observed. IgY antibodies can be produced against many different antigens including pathogens, that is why they can be used as a specific immunotherapeutic agent against bacteria, e.g. as a complement or replacement of antibiotics. This is a very attractive approach especially because of the emerging antibiotic resistant microorganisms. Passively administrated antibodies can provide rapid and fast protection against diseases unresponsive to antibiotic therapies or for patients with immune system defections who are not able to use conventional treatment or vaccinations.

1.10. Immunoglobulin Y: characteristics and function

IgY antibodies are polyclonal immunoglobulins produced by avian species against pathogens, in order to provide an immunogenic response in newly hatched chicks, in which humoral immunity is still not developed. They are present mostly in serum and egg yolk, which provides nutrition to embryos, and are also the dominant class of immunoglobulin in aves. [115, 116] As a member of Vertebrates, the avian immune system is based on two types: innate and adaptive. The second latter is highly specific to pathogens and can be further divided into cellular and non-cellular response. They are carried by two types of cells: B and T lymphocytes, respectively. In the cellular response, T cells control the activity of macrophages, T-helpers and B-cells against the antigen recognized as pathogenic. In non-cellular response, also called humoral, B-cells produce antibodies which circulate in blood and plasma, and can protect organism from pathogen by: agglutination, activating opsonization, activation of cell-mediate cytotoxity or neutralization. B-cells are produced in yolk sack, bone marrow and embryonic liver, and mature in the Bursa of Fabricius, a very unique primary avian lymphoid organ. When B-cells are fully developed, they migrate with the blood to the spleen, tonsils, bone-marrow, thymus and Harderian gland. B-cells activated by T-helper cells turn into plasmocytes and secrete highly antigen-specific immunoglobulins. [117–119] High adaptability and diversity of immunoglobulins in birds is provided by gene hyper conversion, V-J flexible joining and somatic point mutations, as in mammals, but with some differences. In avian antibodies, the heavy and light chain loci possess only one functional V gene, and,

additionally, a family of 25 pseudo-V genes without a transcriptional and recognition signal. At the beginning of a bird life, in process of gene conversion, base pairs from pseudo V-genes replace homologues sequences in functional V gene. As a result, the immune system can generate a huge repertoire of different immunoglobulins. This process occurs around two weeks after the B-cells are transferred to the Bursa of Fabricius to become mature. When a bird is older diversification of immunoglobulins is maintained by somatic hypermutation. [116, 120]

Three classes of immunoglobulins in birds are known: IgY, IgM and IgA. When the egg is being formed, developed IgYs are transfered with the mother's blood to the egg yolk. It proceeds by a FcRY-receptor-dependent transport from the yolk sac membrane through the yolk and further to the blood of the embryo. This transport is also pH-dependent. At pH 6, IgYs are bound by FcRY receptors in the egg yolk when they trans-pass the membrane. These complexes release IgYs when they reach the blood, where pH is 8. [121, 122] At the later stages of egg development, other types of antibodies such as IgMs and IgAs are transferred from the oviduct and deposited into the egg white. [123] Fig. 13 shows a schematic picture of different avian immunoglobulins.

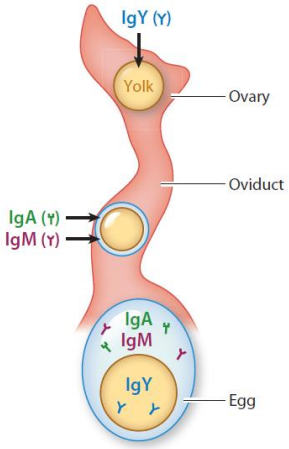


Fig. 13. A scheme of avian immunoglobulins distribution during egg development. [123]

At the beginning, IgYs have been called as IgGs, due to similarities in their function and origin (IgY is a progenitor of IgG). It has changed due to Leslie and Clem et al. in 1969 who differentiated IgYs from IgGs. [115] Differences in their molecular structure and properties can

be clearly seen e.g. IgYs do not contain a well-defined hinge region, possesses four constant domains (instead of three like in IgGs) in addition to the variable domain, IgYs are more hydrophobic because of their bigger hydrophobic Fc region, their isoelectric point is between 5.7 and 7.6 (for IgG 6.1-8.5) and it has bigger weight (IgY ~180 kDa, IgG ~160 kDa). There are also differences in biological functions: compared to IgGs, IgYs do not provoke the mammalian complement system, do not interact with mammalian Fc receptors, do not bind to rheumatoid factor, neither to protein A nor G. [116, 119, 124–126] Fig. 14 shows the differences between an IgG and an IgY.

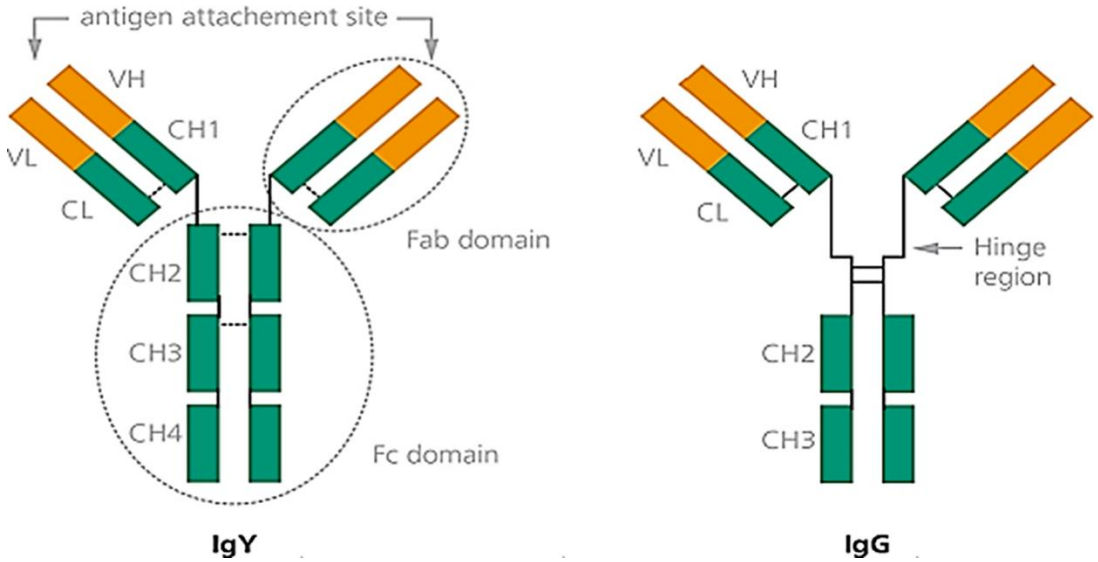


Fig. 14. A scheme of an IgY and an IgG showing the main structural differences. [119]

1.11. IgY technology

IgY technology is the production of specific polyclonal antibodies in birds (mostly in chickens), using their natural way of maternal passive immunity transition to offspring. This approach is relatively cheap, effective, simple and more ethical than using mammalian system. It is officially recognized as the alternative method for antibody production supporting the animal welfare. [127] IgYs are widely used in commercial and research applications, including the fields of diagnostic and proteomics. [128] Although the most valuable and promising IgY

application is to use it to treat and prevent human and animal diseases. [123] The first step includes the immunization of chickens with chosen antigens. The IgYs produced as a response, are transported with the blood to the egg and accumulate in egg yolk in high quantities. As a next step, IgYs are purified and can then be administrated orally to the host organism/ patient, where they can function as an acquired passive immunity against the specific pathogen their development was based on. The whole process of IgY production takes 4-8 weeks. The entire procedure is shown in Fig.15.

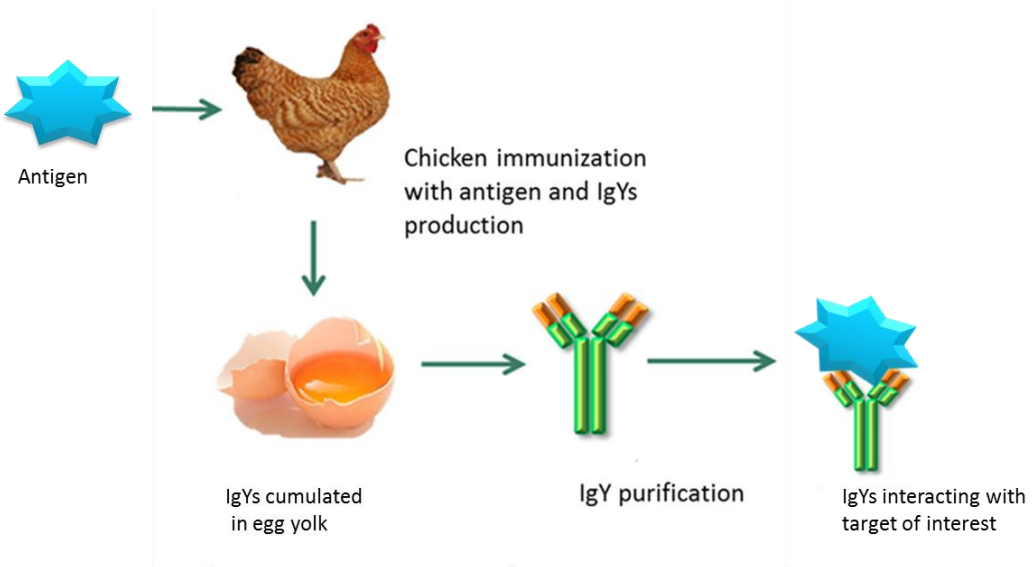


Fig.15. Development of specific IgYs in chickens consist of several stages: 1) identification of antigen (pathogen), 2) immunization with antigen in several cycles to increase the final concentration of IgYs, 3) development of IgYs and transfer to egg yolk, 4) purification of IgYs from other egg components, 5) final batch of specific to antigen IgYs. [18]

There are many advantages of using avian IgY antibodies: 1) easy purification (only one class of IgY antibodies in egg yolk and high IgY concentration), 2) polyclonal, so its specificity to antigen will not decrease significantly after the change of single epitopes, 3) can be produced against conserved mammalian proteins, 4) do not recognize mammalian epitopes, 5) method of production is not harmful to animals (collecting eggs instead of blood), 6) can be used in passive immunization. [119, 123, 129, 130] IgYs are accumulated in egg yolk in high

doses - 1 ml contains up to 25mg of IgYs. Additionally, IgYs show high stability at temperatures ranging between 30 – 70 °C, and in pH 3.5 – 11. [18, 129]

Chicken are immunized with the antigen of interest in the dose between 10µg and 1mg, starting usually with the initial immunization followed by three booster doses in time intervals to obtain higher antibody titers. There are different methods of an antigen introduction: subcutaneous, intramuscular, intravenous or oral. Intravenous rout can cause anaphylactic reaction, has to be introduced very slowly and should be used without adjuvants. Among all the methods, the highest antibody titers are achieved by intramuscular injections. [118, 131–133] First specific IgYs can be already found in serum after 6-7 days. [134]

An optimal dose of antigen depends on its size and type and has to be experimentally tested. For example it is advised to use carriers e.g. BSA or KLH to couple small antigens like short peptides below 10kDa, to enhance the immune response. When immunization is done with an antigen bigger than 50kDa, obtaining 2-10% specific activity of IgYs can be expected. Unfortunately, a drop down of specificity to less than 1% can be observed, when antigen has lower molecular weight. [116]

Additionally to antigen, modulators stimulating B-cells can be used, which improve the immune response in chickens e.g. Freund´s complete adjuvant- mycobacterial antigens emulsified in mineral oil (FCA), Freund´s incomplete adjuvant – water and oil emulsion (FIA), specol – water with purified mineral oil or lipopeptide Pam3Cys-Ser-(Lys)4 (PCSL). FCA is the most popular and most effective one. [116, 135, 136]

One hen is able to produce up to 150mg IgYs per egg. When this number is combined with average number of eggs hen can lay per year – 325, it can give a result of 40g of IgYs per year. When compare with mammals- 200mg of IgGs per bleed giving 1.4g of antibodies per year. [130, 137–139] A comparison between production of polyclonal antibodies in chickens and mammal is shown in Table 4.

There are different methods of IgY purification depends on scale, costs, effectiveness and technology: 1) precipitation with e.g. salt, dextran/sodium/ammonium sulphate, xanthan, ethanol, polyethylene glycol (PEG), 2) chromatography and 3) ultrafiltration. All these methods are summarized in Schade et al. 2005. [116] Usually, in order to obtain higher purity or ratio of specific IgYs, methods are mixed e.g. precipitation is followed by affinity chromatography.

Tab. 4.[130]

Parameter	Mammal IgG	Chicken IgY
Source of antibody	Blood serum	Egg yolk
Antibody sampling	Bleeding (invasive)	Collecting eggs (non-invasive)
Antibody amount	200mg/bleed (40 ml blood)	100-150mg/egg (5-7 eggs per week
Amount of antibody per year	1,400mg	40,000mg
Amount of specific antibody	~5%	2-10%

1.12. IgYs against pathogenic bacteria

The choice of an antigen depends on the characteristics of the pathogen and therapy strategy. IgYs can be generated against colonization factors (outer membrane proteins, fimbriae/pili and lipopolysaccharides), flagella, mucosal receptors, enzymes and toxins important for bacterial survival. [140, 141] Wherefore, several strategies for using IgYs in host protection can be distinguished: 1) agglutination of bacteria, 2) inhibition of bacterial adhesion, 3) suppression of virulence factors, 4) toxin neutralization and, 5) enzyme inactivation. [142] They are shown on Fig. 16. The process of chicken immunization can be influenced by the following factors: antigenicity of the immunogen, type of adjuvant, route of antigen delivery, frequency of administration, avian properties (breed, age, egg lying capacity). [116]

IgY technology is an effective way to provide immunity against a wide range of pathogens, which can reduce or possibly replace the use of antibiotics in clinics and industry, and provide successful prevention, treatment, or growth enhancement overtaking the problem of increasing antibiotic resistance. An increased number of reported IgYs against a wide range of bacteria can be observed. Generated IgYs against anti-cell-associated glucosyltransferase (anti-CA-GTAase) of *S.mutans* can selectively suppress oral colonization of those microbes. [143–145] Specific IgYs inhibit the adherence of *S.mutans* by 59%, but only 8% when IgYs from non-immunized hens were used, which shows that anti *S.mutans* IgYs could be used to prevent dental plaque in humans. [145] Anti-*P.gingivalis*-IgYs can be used as prevention for periodontitis.[146] *In vitro* studies on human intestinal epithelial cell culture

Caco-2, show that *S.enteritidis* incubated with specific IgYs, lose their adherence to human cells what inhibits the bacterial infection. [18, 147]

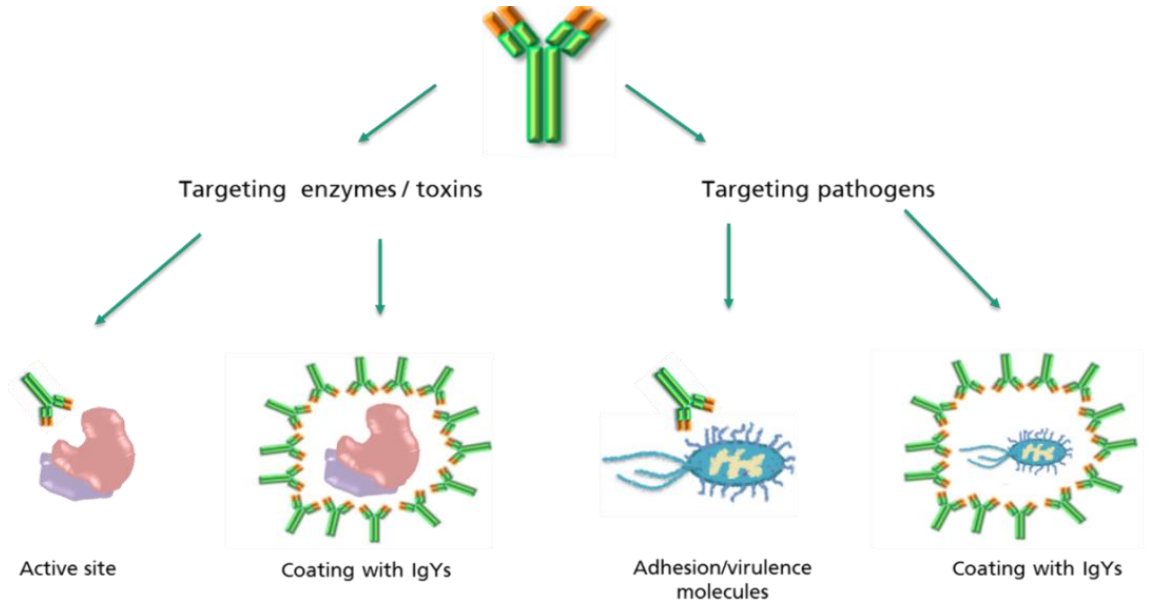


Fig.16. Four different strategies to target pathogenic bacteria with IgYs. [18] Immunoglobulins Y can be developed against enzymes/toxins expressed by bacteria and neutralize them by blocking their active site or building a kind of coat all over their surface. IgYs can also target microbes directly by blocking their adhesion/virulence molecules or coat their surface what suppresess the biological functions of bacteria.

Nilsson et al. made a 12 year study on prophylactic oral IgY treatment against *P.aeruginosa* on 17 patients and reported in most cases prevention of its colonization, which indicates the high potential of IgYs as a prevention for respiratory infections caused by this bacteria. [148] Therapeutic strategies based on IgYs, generated against bacterial enzymes/toxins also bring promising results. LeClaire et al. and Trott et al. reported that IgYs specific to enterotoxin B and botulinum type A, neutralize their activity and therefore can prevent and treat infections of *Staphylococcus aureus* and *Clostridium spp*., respectively. [149, 150] Hirai et al. passively immunized mice with 3 different types of IgYs specific to *V.cholerae*: anti-01, O139 and anti-cholera toxin B and reported effective prevention of cholera infection. [151] Another example is anti-UreC-IgY, which was generated against one of the subunits of

Urease enzyme and successfully prevented and eradicated antibiotic resistant *H.pylori* infections causing gastritis, gastric ulcer and gastric cancer. [140, 152] Added as a supplement to food, for example in yogurt, IgYs can be used as a widely available prevention and treatment for humans and animals against *H.pylori*. [153, 154] IgYs against *E.coli* 0157:H7 also have high potential to be a food additive for passive immunization protecting humans from harmful results of its infections: diarrhea, hemorrhagic colitis and hemolytic uremic syndrome.[155]

IgYs can be used as well in farm animals for prevention and as an alternative treatment in the form of a food additive against common bacterial livestock diseases. Li et al. reported Anti-K88+ IgYs successfully prevented *E.coli* infection in pigs. [156] The growth of *E.coli* in cattle was inhibited by Anti-O111 IgYs and increased uptake by macrophages was observed. [157] Yokoyama et al. orally administrated specific IgYs against *S.typhimurium* and *S.dublin* to neonatal calves, which prevented salmonellosis in animals. [158] Gurtler et al. reduced salmonellosis of eggs from previously infected chickens by oral therapy with anti-*S.enteritidis*-IgYs. [159] Moreover, Zhen et al. studied the efficacy of IgYs against bovine mastitis caused by *S.aureus* and compared it with antibiotic therapy and reported that usage of IgYs for clinical and experimental bovine mastitis showed cure rates of 50% and 83.3%, respectively. In comparison to penicillin the cure rates were 33.3% and 66.7%, respectively. [160] Another example is research done by Tsubokura et al. on IgYs against *C.jejuni* as a prevention and therapy for infected chickens. Prophylactic efficacy was observed as a 99% decrease of number of bacteria, and therapeutic efficacy as an 80-95% decrease number of faecal bacterial counts. [18, 161] IgYs generated against different bacterial strains are summarized in Table 5.

Tab. 5. Examples of IgYs generated against different antibiotic resistant bacterial strains. [18]

Pathogen	Target	IgY therapy	Reference
Streptococcus mutans	humans	Inhibition of bacterial adherence and prevention of dental plaque	[145]

Helicobacter pylori	humans	Anti-UreC-IgYs inactivate Urease and eradicate *H.pylori* colonization	[140]
Escherichia coli	humans	IgY Inhibits the growth of *E.coli* O157:H7 strain	[155]
	pigs	Anti-K88+-IgYs protect against *E.coli* and enhance weight	[156]
	cattle	Anti-O111-IgYs inhibit growth of *E.coli* and activate uptake by macrophages	[157]
Salmonella spp.	Cattle	IgY against *S. typhimurium* and *S. Dublin* protects neonatal calves from infection and lethal effect	[158]
	humans	IgYs generated against *Salmonella Enteritidis* protects infected cultured human intestinal epithelial cells by inhibiting the bacterial adhesion	[147]
	chickens	Anti-*Salmonella Enteritidis* IgYs reduce contamination of eggs from previously infected chickens	[159]
Pseudomonas aeruginosa	humans	Anti-*Pseudomonas aeruginosa* IgYs prevents infections in cystic fibrosis patients	[148]
Staphylococcus aureus	humans	Anti-enerotoxin B-IgYs protected monkeys from lethal effect of *S.aureus* toxin	[149]
	cattle	Anti-*Staphylococcus areus* IgYs reduced symptoms of mastitis caused by *S.aureus*	[160]
Clostridium spp.	humans	IgYs generated against enterotoxin A block its activity *in vivo a*nd IgYs against *Clostridium perfringens* inhibit growth of its vegetative cells or spores	[150] [162]

Campylobacter jejuni	chickens	Campylobacter jejuni specific IgYs protects chickens from infection and decreases already existing Campylobacter jejuni infections	[161]
Vibrio cholerae	humans	Anti-01-, O139 and anti-cholera toxin B subunit protected mice from cholera infection	[151]

1.13. Oral Immunotherapy based on IgY

The idea of oral administration of IgYs specific to pathogens would be definitely an attractive approach, especially against pathogens infecting the gastrointestinal track. Advantages of IgYs are summarized in Table 6. There are several human studies on IgY-based therapies e.g. against *S.mutans*, *P.gingivalis*, *H.pylori*, *P.aeruginosa* and *C.albicans* in which it was showed that IgYs have a big potential to prevent or decrease microbial infections. [145, 146, 153, 154, 163, 164]

Eggs as an everyday food product do not cause the risk of toxic side effects. Concerns about allergy reactions are not necessary, as typical egg white allergens like ovomucoid, lysozyme, ovalbumin and ovotransferrin, as well as egg yolk allergens like livetin, phosvitin and apovitillin are not present in an extracted and affinity purified IgYs batch. An IgY itself does not activate the mammalian complement system, does not interact with rheumatoid factors, proteins A and G, nor with mammalian Fc receptors. [116]

Proteins undergo digestion reactions in the stomach and in the intestine, where pancreas secretes its proteolytic enzymes. In the stomach, pepsin breakdowns proteins into peptides and amino acids, whereas hydrochloric acid denatures proteins. In the intestine, two proteases are active: trypsin, which cut peptides at basic amino acids and chymotrypsin which cut peptides at aromatic amino acids. Researchers have investigated also IgY susceptibility to proteolytic digestion by proteases and acids. It was investigated that IgY is more sensitive than IgG to HCl denaturation and its activity drops down rapidly in pH between 3-4. [165–168] IgY might lose its specificity when passing the digestive route through stomach and pancreas. [169, 170] Although, it was observed that IgYs are more sensitive to pepsin digestion

compared to IgGs, they are less susceptible to proteolytic activity of trypsin and chymotrypsin. [165, 171] IgY stability is still maintained in high temperatures up to 60-70°C, moreover no decrease in IgY concentration was observed after cooking 6 min in 100°C. [18,165, 166, 168]

Despite concerns about the inactivation of IgYs during the digestion process, it is observed that orally applied IgYs against pathogens still give promising results (chapter above). The stability of IgYs in harsh conditions can be increased by additives and different encapsulation methods. For example, added sorbitol can suppress the inactivation of IgY in low a pH. [167] Similar effects can be observed in the presence of sucralfate and sucrose. [172, 173] Encapsulation using gelatin or chitosan-alginate capsules, methacrylic acid copolymer also prevent from proteolytic digestion and denaturation, keeping preserving IgY activity. [156, 174–176]

Tab. 6. Advantages of IgYs. [18]

• Specific antibody ready to be delivered into patient/organism of choice
• Do not provoke allergic reactions
• Cost-effective and convenient production
• High yield (up to 25mg of IgYs per ml of egg yolk)
• Stability (pH 3,5-11, 30-70°C)
• High avidity
• Low cross reactivity (in comparison to other polyclonal antibodies)
• Method of production is not harmful for animals
• Can be produced against mammalian proteins

1.14. Bioinformatic tools in the antibacterial immunotherapies design

The main goal of bioinformatics is transfer, analysis and interpretation of the data resulted from experiments and study on genome, transcriptome and proteome. It combines fields such as biology, computer and mathematical sciences in order to store, maintain and translate the information generated in biotechnology, genetics and molecular biology. [177]

In the development of a new immunotherapy against pathogens, *in silico* analysis of the bacterial proteome is essential. The most favored candidates as potential therapeutic antibody targets are molecules exposed on the bacterial surface or secreted proteins with virulence properties. *In silico* work mainly focuses on the identification of genes encoding these molecules and proteins, protein sequence analysis and algorithm-based prediction of antigenicity of full or fragmented molecules chosen for the antibodies development. [178]

The fundaments in this field of research are publicly available databases of: complete bacterial genome sequences, protein sequences, function and active sites, as well as 3D macromolecular structures. Examples of such a data collections are as follow: NCBI, the National Center for Biotechnology Information https://www.ncbi.nlm.nih.gov/ , comprises data of genomes, genes, proteins and chemicals, including genome sequences and assemblies, mapped variations and markers, information from epigenomic studies, nucleotide sequences, sequence clusters, tools for the study of gene expression, protein sequences and structures, protein functional domains, catalog of chemical bioactivity, molecular pathways linked to activity of chemicals. [179]

UniProt database http://www.uniprot.org/ includes literature protein information such as name, amino acid sequence, sequence clusters, biological function, interaction with different molecules, but also different variants and proteomes of different organisms.[180]

RCSB Protein Data Bank www.rcsb.org is a source of information about 3D structure of proteins, nucleic acids and complex assemblies, based on X-ray crystallography, Cryo-EM and NMR spectroscopy. [181] Every protein has its unique UniProt and PDB identification number, additionally EC number when protein has enzymatic functionality. With these IDs a detail definition of protein structure, active sites, substrate specificity, single amino acid residue function and their mutability is possible. There are several databases providing these information e.g. Catalytic Site Atlas https://www.ebi.ac.uk/thornton-srv/databases/CSA/ or HotSpot Wizard 1.7 https://loschmidt.chemi.muni.cz/hotspotwizard-1.7/. [182, 183] Moreover, there are some informatic tools for individual analysis, modeling and molecular visualization e.g. UCSF Chimera https://www.cgl.ucsf.edu/chimera/ or Jmol http://jmol.sourceforge.net/. [52, 184] In the choice of the best antigen for antibody development, immunogenicity or hydrophobicity (more hydrophobic proteins are less antigenic) parameters analysis is very important. Helpful are programs such as e.g.: Peptide

Property Calculator http://biotools.nubic.northwestern.edu/proteincalc.html or ProtScale https://web.expasy.org/cgi-bin/protscale/protscale.pl . [185, 186]

More database and bioinformatics tools depends on the research need or project nature can be found e.g. on ExPAsy – Bioinformatic Resource Portal https://www.expasy.org/. [187]

Aims

2. Aims

2.1. General aims

In times of a worldwide increasing problem with fast developing new resistant bacterial strains and less and less effective antibiotic therapies, the perspective of establishing a universal IgY-based immunotherapeutics targeting ESBL-producing bacteria is a very promising alternative. Therefore, the aim of this study was to create a coherent system based on already established biological and chemical components, to prove the concept that specific IgYs designed with the help of *in silico* tools can be a good candidate for an oral passive immunotherapeutic against ESBL-producing gram negative bacteria. Another aim was to show that it is possible to inhibit the growth of these bacteria using different IgY-based strategies: IgYs targeting bacterial cell surface and IgYs targeting ß-lactamase. As the family of ESBLs constantly grows and there is lack of their clear classification in the literature, this study intended to develop a model based on ESBL parental enzyme TEM-1 ß-lactamase (class A) and TEM-1 producing *E.coli* strain. Subsequently, to use this model to evaluate the properties of in-house designed specific IgYs. The project was divided into 3 sections.

2.1.1. Analysis of TEM-1 by bioinformatic approaches

The main aim of this part was to analyze the amino acid sequence and conformation of ß-lactamase TEM-1 in comparison to TEM- type ESBLs using different bioinformatic tools, in detail to identify active site and conservative residues. Based on this information, another aim was to design *in silico* short peptides mimicking amino acid sequence of TEM-1 active site.

2.1.2. Antigen preparation for chicken immunization and IgY development

In this section the aim was to develop different IgYs, according to designed strategies for an oral passive immunization against TEM-1 producing *E.coli*, as a potential complement or as a replacement for the existing antibiotic therapy. Chosen strategies were as follow: a) IgYs as a complement to antibiotics - inactivation of TEM-1 by targeting the whole enzyme or

inactivation of TEM-1 by targeting the active site, b) IgYs as a replacement for antibiotics - neutralization of bacteria by targeting surface molecules. The objective of this part was to prepare different antigens for the chicken immunization in order to develop IgYs against: whole TEM-1 protein, short peptides mimicking active site, inactivated TEM-1-producing *E.coli*.

Another aim was to develop the most effective and specific IgYs, therefore these targeting the enzyme were extracted independently at two different levels: purified by precipitation methods and affinity purified, and these targeting bacteria were developed separately against E.coli inactivated by two different methods: heat and e-beam inactivation, and affinity purified.

2.1.3. Characterization and functional studies on IgYs developed against TEM-1 producing *E.coli*

In this section the aim was to compare IgYs with IgG by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Furthermore, to investigate the specificity and activity of developed IgYs against different targets *in vitro* by ELISA and inhibition assay. Additional objective was to visualize the binding of generated IgYs to a target, using the immunofluorescence staining method.

Materials and methods

3. Materials and methods

3.1. Study design

Two main strategies using specific IgYs were designed in this study to inhibit the growth of TEM-1 producing *E.coli*. The first strategy was to inactivate the TEM-1 by specific IgYs and kill the *E.coli* in the presence of ampicillin (described in subchapter 2.1.2 as a complementary strategy). For this, the two types of IgYs were generated: 1) targeting the active site, developed by immunization of a chicken with a short peptide synthesized *de novo* resembling the aminoacidic sequence surrounding critical catalytic residues of TEM-1 analyzed with bioinformatic approaches, 2) targeting the whole TEM-1 structure , developed by immunizing the chicken with purified recombinant TEM-1 protein. The second strategy was to eradicate the TEM-1-producing *E.coli* by inhibiting their biological processes with IgYs specific to the bacterial cell surface (described in subchapter 2.1.2. as an alternative strategy to antibiotics), which were generated by immunization of the chicken with inactivated whole cells of TEM-1-producing *E.coli*. Both strategies are shown on the Fig. 17.

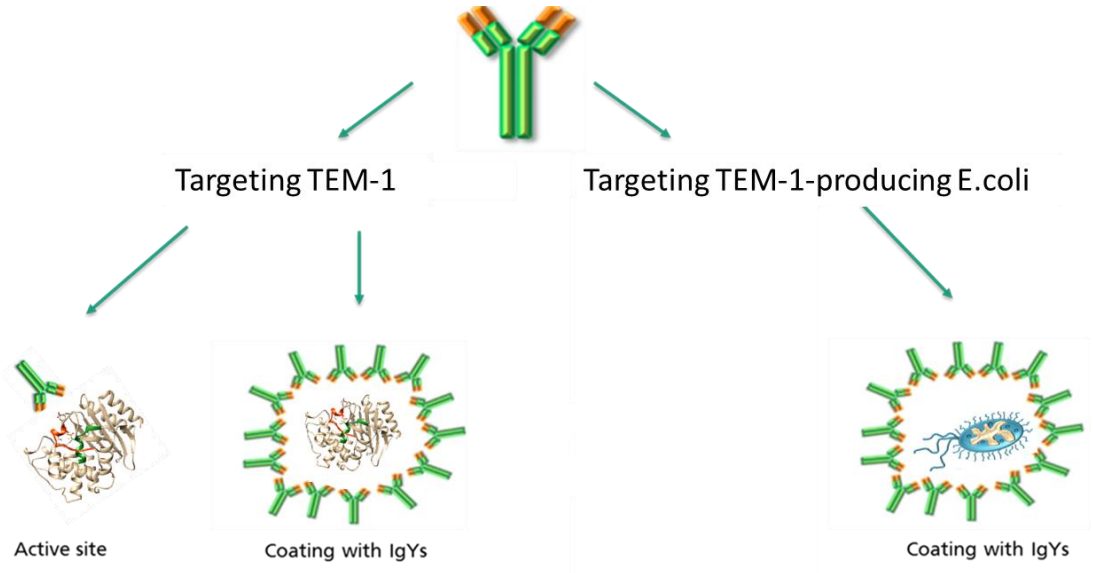


Fig. 17. Two strategies based on specific IgYs designed and developed in this study against TEM-1- producing E.coli.

3.2. Bioinformatic approach in target analysis for IgY development

3.2.1. Analysis of TEM-1

Amino acid sequence analysis of TEM-1 protein was performed using UniProt [180] (access id P62593) and RCBS PDB [181] (access id 1axb and 1btl). The catalytic sites and conservative residues were identified using Hydrolases Catalytic Sites database [188], Catalytic Site Atlas [182] and HotSpot Wizard [183], additionally complemented by analysis of Lahey ß-lactamase data base. [34] Protein visualization was performed by using Chimera software. [52] The summary of information of TEM-1 is shown in table 7.

Tab.7.

E.C. name	**Beta lactamase TEM-1**
E.C. number	3.5.2.6.
Source organism	Escherichia coli
Weight	28,98kDa
Length	263 amino acids
Residues	UniProt: 24-286, PBD: 26-288
UniProtID	P62593
PDB Entry	1axb, 1btl

3.2.2. *In silico* design of short peptides for IgY development

Based on the bioinformatic analysis of active site of TEM-1 (described above), two 11-amino-acid-long sequences containing specific conserved catalytic residues were selected for peptide synthesis in order to mimic the active site as antigens for specific IgYs development. The sequences chosen were as follow: 1- RFPMMSTFKVL and 2- TRLDRWEPELN. Visualization of amino acid sequences 1 and 2 within TEM-1 as well as visualization of catalytic residues was performed by using Chimera software. [52] Antigenic properties of chosen sequences were tested by Antigen Profiler Peptide Tool (Thermofisher online tool, https://www.thermofisher.com/de/de/home/life-science/antibodies/custom-antibodies/custom-antibody-production/antigen-profiler-antigen-preparation.html).

3.3. Bacterial strain and culture

3.3.1. Cultivation conditions

A strain of the TEM-1 expressing *E.coli* BW25113 *ΔbamBΔtolC* where TEM-1 gene was integrated behind the *bla* promoter was a gift from Prof. PhD. Gerry Wright and Prof. PhD. Brian Coombes (Michael G. DeGroote Institute for Infectious Disease Research, McMaster University, Canada). Bacteria were cultured in Luria Bertani Broth LB medium (28713, Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) with 0.1mg/ml ampicillin (11593027, Life Technologies GmbH, Darmstadt, Germany) for 18h, at 37˚C and with 150rpm. Streak plates were prepared by spreading the bacteria on LB-agar plate (19344, Sigma Aldrich Chemie GmbH) containing 0.1mg/ml ampicillin by sterile loop. Plates were incubated for 16h, at 37˚C and then kept at 4˚C. A single colony was picked to inoculate 5 ml of LB medium (with 0.1 mg/ml ampicillin). The inoculation was incubated overnight on a shaker (37˚C, 150 rpm), transferred to a sterile conical flask containing 200ml of LB medium prewarmed to 37˚C (with 0.1 mg/ml ampicillin) and kept on a shaker until bacteria reached OD_{600} ~0.4. Glycerol stocks were prepared by mixing liquid bacteria culture 1:1 (vol/vol) with 50% Glycerol (G5516, Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) solution diluted with MiliQ water, and kept at -80˚C. The quantitive analysis of E.coli was done using a spread-plate method and establishing a bacterial growth curve.

3.3.2. Colorimetric detection of TEM-1 in E.coli cell culture

E.coli was cultivated as described in subchapter 3.2.1., transferred into tubes and kept on ice. Half of the samples were sonicated (2x cycles, 1min, power 70%), while the other half was left untreated. Next, the treated and untreated bacteria culture samples were diluted 1:2.5 (vol/vol) with PBS (P4417, Sigma Aldrich GmbH, Taufkirchen, Germany) transferred in triplicates to 96 well plate and mixed 8:1 (vol/vol) with 0,5mg/ml Nitrocefin (2388-5, BioVision Inc. Milpitas, CA, USA) dissolved before in 0.1M PBS and DMSO (D4540, Sigma Aldrich GmbH, Taufkirchen, Germany) 200:1 (vol/vol). As controls, LB medium with 0.1mg/ml ampicillin and PBS were used, also mixed with Nitrocefin solution. Samples were incubated in room temperature for 30min, then photographed and measured for absorption in a plate reader at 486nm.

3.4. Antigen preparation

3.4.1. TEM-1 and synthesis of short peptides p1, p2

As an antigen for the development of TIgY and aTIgY against TEM-1 produced by *E.coli*, the Recombinant *E.coli* Beta lactamase TEM precursor protein was used (Ab67672, Abcam plc, Cambridge, UK). For the development of p1IgY and p2IgY/ap2IgY, amino acid sequences 1 and 2 (see 3.2.2) were synthesized *de novo* by Gallus Immunotech Inc., Fergus, ON, Canada and used as antigens. Short peptides sequences were: p1-RFPMMSTFKVL and p2-TRLDRWEPELN. Additionally, they were conjugated with KLH carrier through the thiol group of an additional Cys residue on -C end of the peptide to increase the immunogenicity of peptides. Peptides were purified on HPLC (p1- 96.8%, p2- 95.7% purity), tested on MS (Mw p1= 1459.86 g/mol, p2= 1531.72 g/mol) and lyophilized. Short peptide for the development of ap2IgY was synthesized by Davids Biotechnologie GmbH, Regensburg, Germany and was as follow: p2´ – TRLDRWEPELN, conjugated with KLH carrier through the Thiol group of an additional Cys residue on -C end of the peptide. The peptide was purified on HPLC (95% purity), tested on MS (Mw p2´= 1531.72 g/mol) and lyophilized.

3.4.2. Whole bacterial cells antigens

E.coli was cultivated as described in subchapter 3.2.1 until OD_{600} reached ~0.6. Cells were harvested by centrifugation for 10min at 4˚C, at 4000g washed twice with PBS and, subsequently, resuspended in PBS to obtain $OD_{600} \leq 3$. Bacteria were inactivated independently by two methods: e-beam (based on Low-Energy-Electron Irradiation) and heat inactivation. The e-beam inactivation of *E.coli* samples was performed as described by *Fertey et al*. using ionizing radiation dose of 5kGy (EBLab 200, Comet, Flamatt, Switzerland).[189] For the heat inactivation, bacteria suspensions were incubated for 1h at 65˚C. Viability tests were performed by applying inactivated samples and control sample (untreated) on LB agar plates, subsequently incubated overnight at 37˚C. To fix and preserve cell membrane structures for subsequent immunization procedures, the heat and e-beam inactivated samples were treated with the Membrane Structure Conservation kit (K105, Davids Biotechnologie GmbH, Regensburg, Germany) according to manufacturer's instruction. Each sample with different whole bacteria cell antigens contained ~8x10^8 CFU.

3.5. Chicken immunization and IgYs purification

3.5.1. Generation of IgYs against TEM-1 and active site of TEM-1

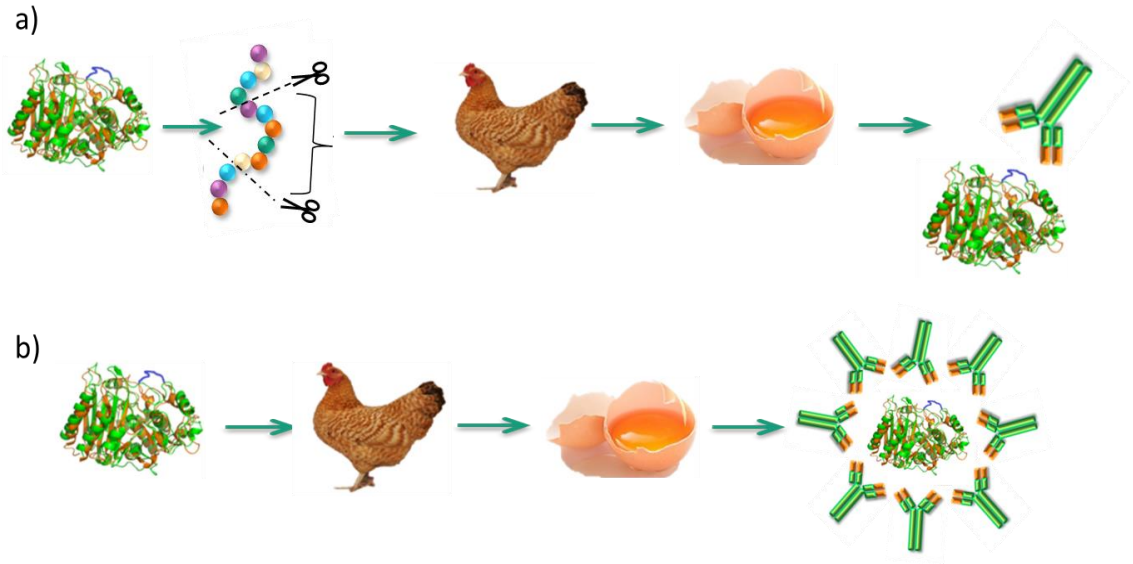


Fig. 18. A scheme of specific IgY generation against a) the active site of TEM-1 ß-lactamase, which is based on immunization of chickens with *de novo* synthesized short peptides mimicking amino acid sequence of the active site, b) the whole TEM-1 ß-lactamase, which is based on chicken immunization with a whole protein (the Recombinant E.coli Beta lactamase TEM precursor protein).

3.5.1.1. Generation of p1IgY, p2IgY and TIgY.

Immunization and purification of p1IgY, p2IgY and TIgY was customized and performed by Gallus Immunotech Inc., Fergus, ON, Canada. Chicken were immunized separately with purified short peptides p1, p2 and TEM precursor protein (shown on Fig. 18) in 4 2-week-long intervals, starting with primary immunization (1mg of antigen p1/p2/ TEM precursor protein in Freud`s complete Adjuvant) and 3 boosters (three times 0.33mg antigen p1/p2/ TEM precursor protein in Freud`s complete Adjuvant). After 2 weeks eggs were collected and IgYs were purified from egg yolk by a two-step precipitation, then diluted in PBS pH 7.3 containing 0.075% sodium azide. Three IgY fractions were obtained: p1IgY (28.4mg/ml), p2IgY (30.8mg/ml) and TIgY (27mg/ml).

3.5.1.2. Generation of ap2IgY and aTIgY with affinity purification step

One of the methods to obtain a higher fraction of specific antibodies is affinity purification, which is used as additional step to standard precipitation protocols. Immunization and affinity purification of ap2IgY and aTIgY was customized and performed by Davids Biotechnologie GmbH, Regensburg, Germany. Chicken were immunized separately with purified short peptide antigen p2′ and the TEM precursor protein (shown on Fig. 18) in 4 2-week-long intervals. Eggs were collected after 10-15 days after the last immunization, egg yolk was separated, IgYs were precipitated and specific IgYs were affinity purified using the antigen (p2' or TEM precursor protein) immobilized on the resin. IgY fractions were diluted with PBS containing 0.02% sodium azide to the final concentration of: ap2IgY (0.41mg/ml), aTIgY (0.35mg/ml).

3.5.2. Generation of eIgY and hIgY against TEM-1 producing E.coli with affinity purification step

Immunization and affinity purification of eIgY and hTIgY was customized and performed by Davids Biotechnologie GmbH, Regensburg, Germany. Chicken were immunized separately with prepared whole bacterial cell antigens (shown on Fig.19): *E.coli* inactivated by e-beam and heat in 4 2-week-long intervals. Eggs were collected 10-15 days after the last immunization. The egg yolk was then separated, IgYs were precipitated and specific IgYs were affinity purified using the antigen immobilized on the resin (each type of inactivated bacteria cells). IgY fractions were diluted with PBS containing 0.02% sodium azide to the final concentration of: eIgY (0.51mg/ml) and hIgY (0.48mg/ml).

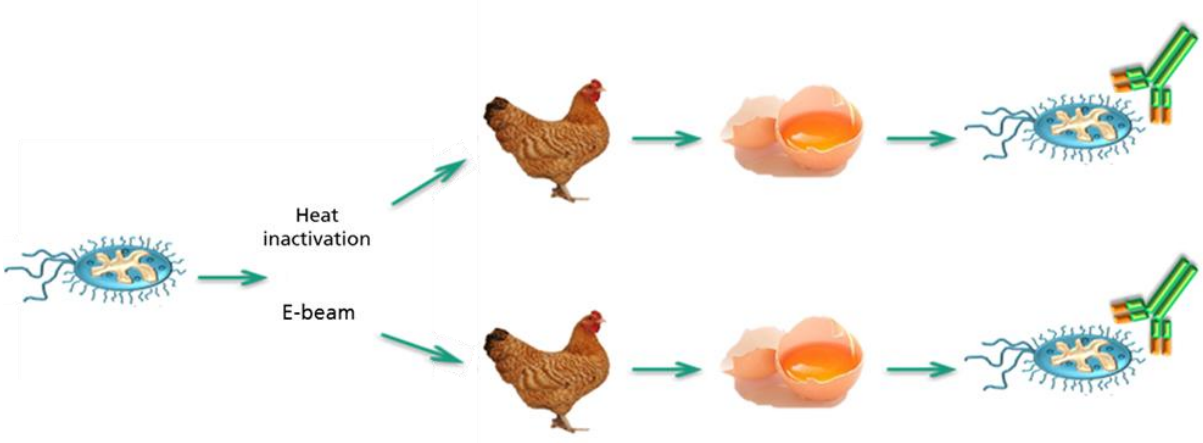


Fig. 19. A scheme of specific IgY generation against *E.coli* cell surface proteins which is based on immunization of chickens with *E.coli* whole cell antigens, prepared by inactivation by two methods: heat and e-beam.

3.5.3. Non-specific IgYs: rIgY

In the study, non-specific IgYs were used as a control. They were purified from non- or pre-immunized chickens and were as follow: r^{I}IgY (LOT16IgY014E-pi, Gallus Immunotech), r^{II}IgY (LOT16IgY015E-pi), r^{III}IgY (P075.05, Davids Biotechnologie), r^{IV}IgY (16IgY014K-pi, Gallus Immunotech).

3.6. Characteristics of IgYs

3.6.1. SDS -PAGE: reduced and non-reduced

Comparison of molecular weight, heavy and light chains, and purity between p1IgY, p2IgY, r^{I}IgY, r^{II}IgY, r^{III}IgY, r^{IV}IgY, TIgY and IgG_1 AQP1 (sc-25287, Santa Cruz) was performed by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in reduced and non-reduced conditions using 4-15% Mini PROTEAN TGX Stain-Free Protein Gells (4568085, Bio-Rad Laboratories GmbH, Münich, Germany) and applying 200 V for 30min. SDS-page running buffer was prepared by mixing 1/10 10x TGS running buffer (161-0732, Bio-Rad Laboratories GmbH, Münich, Germany) with miliQ water. For non-reduced conditions samples were mixed 1:1 (vol/vol) with 2x Laemmli sample buffer (161-0737, Bio-Rad Laboratories GmbH, Münich, Germany). For reduced conditions, samples were mixed 1:1 (vol/vol) with 2x Laemmli sample buffer previously mixed 20:1 (vol/vol) with ß-mercaptoethanol. Before loading on the gel, samples were incubated at 95˚C for 5min and cooled down on ice. Two standards were used to estimate the size of immunoglobulins: Precision Plus Protein™ Standards unstained (161-0363, Bio-Rad Laboratories, München, Germany) and Precision Plus Protein™ Standards All Blue (161-0373, Bio-Rad Laboratories, München, Germany). The protein bands were visualized by applying a UV light.

3.6.2. ELISA

3.6.2.1. Binding specificity of TIgY, p1IgY, p2IgY and rIgY to the Beta lactamase TEM precursor protein

To determine the specific activity of TIgY, p1IgY and p2IgY against the Beta lactamase TEM precursor protein and to choose which short-peptide-based IgY (p1 or p2) binds better to the active

site of this protein, an indirect ELISA assay was performed (the scheme is shown on Fig. 20 a). A 96 well plate was coated with 100µl of 1ug/ml the Beta lactamase TEM precursor protein diluted in coating buffer carbonate-bicarbonate buffer (prepared according to manufacturer, pH 9,6, BUF030A, Bio-Rad AbD Serotec GmbH, Puchheim, Germany) and incubated overnight at 4˚C. The plate was washed three times with washing buffer - PBS buffer containing 0.05%(vol/vol) Tween®-20 (P9416, Sigma Aldrich Chemie GmbH, Taufkirchen, Gemrnay) and blocked for 2h at RT with 200µl of blocking buffer-PBS buffer containing 0.05%(vol/vol) Tween®-20 and 2% (w/vol) non-fat milk powder (T145.2, Carl Roth, Germany). Next, the plate was washed three times with washing buffer and thereafter, specific IgYs (TIgY, p1IgY, p2IgY) and non-specific r^{IV}IgY used as a control were diluted to 200µg/ml with diluent buffer-PBS buffer containing 0.05%(vol/vol) Tween®-20 and 0.3% (w/vol) non-fat milk powder, and were loaded by multichannel pipette in three technical replicates per sample on the 96 well plate in serial 2-fold dilutions, mixed 10 times and with a change of tips between each dilution. As the next step, plate was incubated for 2h at RT and washed again three times with washing buffer. Afterwards, 100µl of secondary antibody anti-chicken IgY rabbit IgG with horse radish peroxidase conjugate (SA1-9509, Thermo Fisher) diluted 1:5000 (vol/vol) with diluent buffer and was added to each well and incubated for 1h at 37˚C. Plate was washed again three times with washing buffer, then 100µl of the substrate solution TMB Core+ (BUF062a, Bio-Rad AbD Serotec GmbH, Puchheim, Germany) for HRP-conjugated antibodies was added to each well and incubated for 10min at RT in the dark. The reaction was stopped by adding 50µl of 0.2M sulfuric acid (diluted with MiliQ, 1,09072.1000, Sigma-Aldrich, Taufkirchen, Germany) to each well and absorbance of samples was measured at 450nm. ELISA assay was repeated three times (n=3) and values were plotted.

3.6.2.2. Binding specificity of TIgY, p2IgY and rIgY to TEM-1 in E.coli lysate

After the first selection of IgYs specific to the Beta lactamase TEM precursor protein, the next step was to determine the specific activity of TIgY and p2IgY against ß-lactamase TEM-1 produced by *E.coli* in cell culture. Therefore an indirect ELISA assay was performed using E.coli cell lysate (the scheme is shown on Fig. 20 b). To prepare E.coli cell lysate, bacteria were cultivated as described in subchapter 3.2.1. Then aliquots of cell suspension were sonicated on ice in 2 cycles, 1min, applying 70% of power. Cell lysate was diluted with coating buffer 1:32 (vol:vol) what corresponded to ~$1.2x10^6$ CFU/ml, and used to coat a 96 well plate, by loading 100µl per well. The plate was incubated overnight at 4˚C. Plate was washed three times with washing buffer and blocked for 2h at RT with 200µl of blocking buffer. Next, the plate was washed again three times with washing buffer. Thereafter, specific IgYs (TIgY and p2IgY) and non-specific r^{IV}IgY used as a control were diluted to 200µg/ml with diluent

buffer. The next steps were preceded as described in subchapter 3.6.2.1. ELISA assay was repeated two times (n=2) and values were plotted.

3.6.2.3. Binding specificity of aTIgY, ap2IgY and rIgY to TEM-1 in E.coli lysate

In order to determine the specific activity of affinity purified aTIgY and ap2IgY against ß-lactamase TEM-1 produced by *E.coli* in a cell culture, an indirect ELISA assay was performed using *E.coli* cell lysate (the scheme is shown on Fig. 20 c). To prepare *E.coli* cell lysate, bacteria were cultivated as described in subchapter 3.2.1. Then aliquots of cell suspension were sonicated on ice in 2 cycles, for 1min., applying 70% of power. The cell lysate was diluted with coating buffer 1:25 (vol:vol) what corresponded to ~1.5x10^6 CFU/ml, and was used to coat the 96 well plate, by loading 100µl per well. The plate was incubated overnight at 4˚C. Plate was washed three times with washing buffer and blocked for 2h at RT with 200µl of blocking buffer. Next, the plate was washed again three times with washing buffer. Thereafter, specific IgYs (TIgY and p2IgY) and non-specific r^{III}IgY used as a control were diluted to 80µg/ml with diluent buffer. The next steps were preceded as described in subchapter 3.6.2.1. ELISA assay was repeated three times (n=3) and values were plotted.

3.6.2.4. Binding specificity of hIgY, eIgY, rIgY to E.coli

To determine the specific activity of affinity purified IgYs against inactivated by heat and e-beam TEM-1 producing *E.coli*, such as hIgY and eIgY respectively, an indirect bacterial ELISA assay was performed (the scheme is shown on Fig. 20 d). For this *E.coli* were cultivated as described in subchapter 3.2.1., cells were harvested by centrifugation at 4˚C, for 10min, at 4000g, washed twice with 1PBS and incubated in 4% (vol/vol) formaldehyde in 1xPBS at RT for 20min. Next, the cells were washed three times with PBS. Thereafter, *E.coli* cells were diluted with coating buffer 1:25 (vol:vol) to ~1.5x10^6 CFU/ml, and suspension was used to coat a 96 well plate, loaded 100µl per well. The plate was incubated overnight at 4˚C. As the next step, the plate was washed three times with washing buffer and blocked for 2h at RT with 200µl of blocking buffer. Then, the plate was washed three times with washing buffer and thereafter, specific IgYs (hIgY and eIgY) and non-specific r^{III}IgY used as a control were diluted to 80µg/ml with diluent buffer. The next steps were preceded as described in subchapter 3.6.2.1. ELISA assay was repeated three times (n=3) and values were plotted.

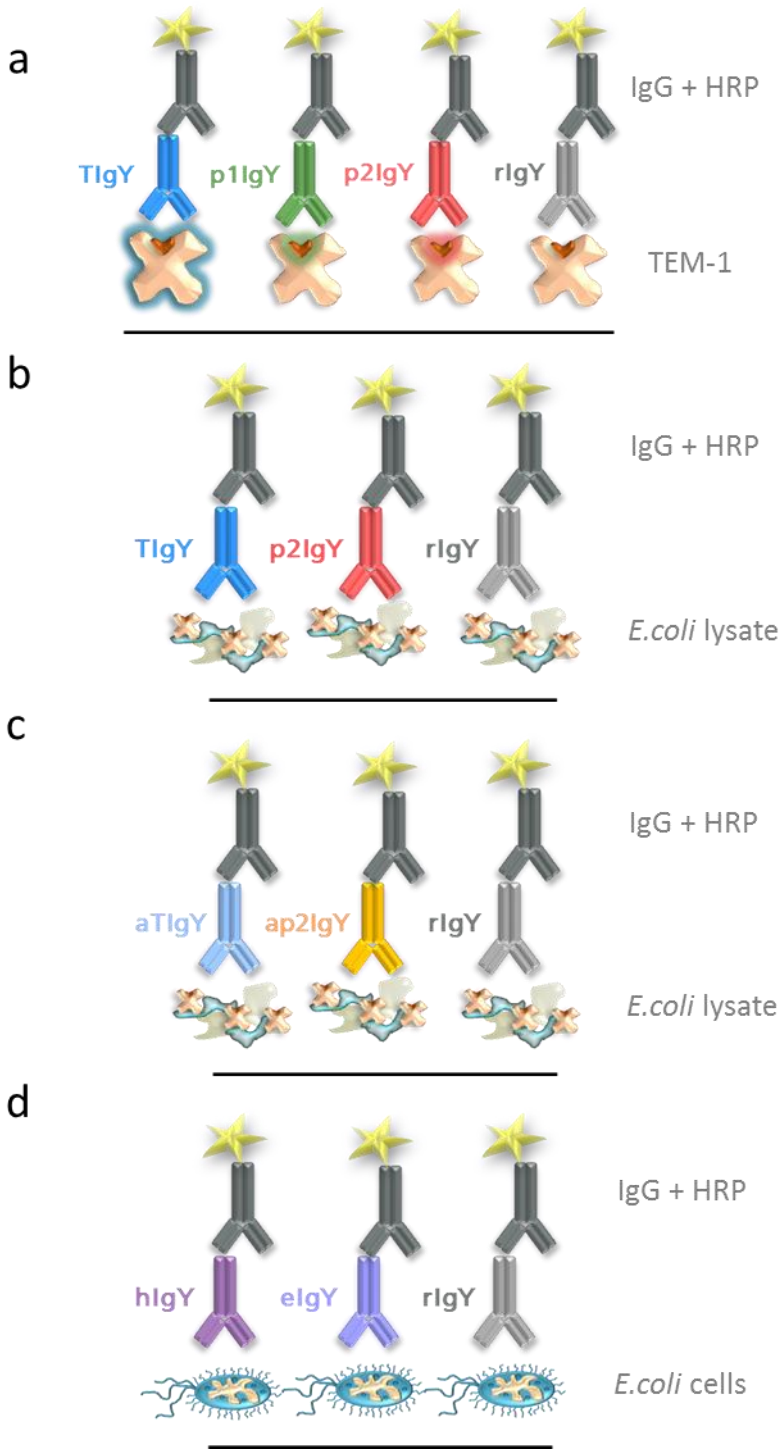


Fig. 20. Different indirect ELISA set-ups to study the binding specificity of developed IgYs: a) TIgY, p1IgY, p2IgY and rIgY to the Beta lactamase TEM precursor protein, b) TIgY, p2IgY and rIgY to TEM-1 in *E.coli* lysate, c) aTIgY, ap2IgY and rIgY to TEM-1 in *E.coli* lysate, d) hTIgY, eIgY and rIgY to bacteria cell surface of *E.coli*. In all studies as a secondary antibodies anti-chicken IgG were used, additionally conjugated with horse radish peroxidase for the binding detection.

3.7. In vitro studies on IgYs

3.7.1. Inhibition assay

To investigate the influence of developed IgYs on growth of TEM-1 producing *E.coli* an inhibition assay was performed, in which *E.coli* inoculates, were incubated for 24h in the presence of p2IgY, TIgY, ap2IgY, aTIgY, hIgY and eIgY. As controls LB medium (no IgY) and non-specific IgYs were used (r^{I}IgY or r^{III}IgY). To compare the growth of *E.coli* after incubation with different IgYs, the drop plate method for colony counting was used (adapted from Chen et al. 2003 and modified). [190] From each mixture 3x 200µl of aliquots was taken using multichannel pipette to prepare drop plates (Agar + 0.1 mg/ml ampicillin) at the time points of incubation: 0h, 3h, 6h and 24h. The 10-fold serial dilutions (10^0-10^6) were made by transferring by multichannel pipette 20µl of mixtures to a well containing 180µl of LB medium with 0.1mg/ml ampicillin, mixing 10 times, repeating the process and changing tips between dilutions. Three replicates of 20µl from each of selected dilutions were plated onto agar plates. Plates with samples from each time point were first incubated for 3h at RT and then incubated for 16h at 37˚C. Colony-forming units were counted to calculate the total number of bacteria colony-forming units per ml of sample for each incubation time point and then were plotted. Additionally, the growth of bacterial cells in mixtures was monitored by bacterial density measurements at OD_{600} at each incubation time point and optically through visual changes. The scheme of the method is shown on the fig. 21 a and 21 b.

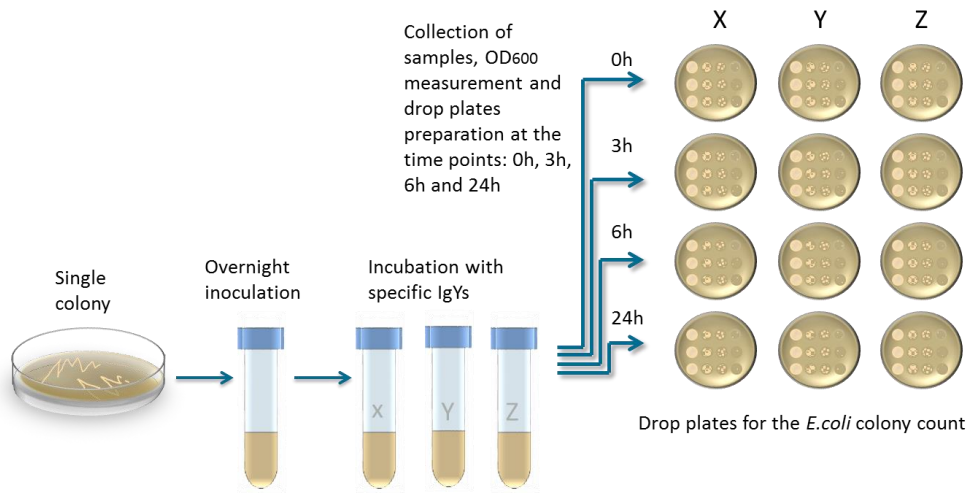


Fig. 21a. A scheme of the inhibition assay and the drop plate method.

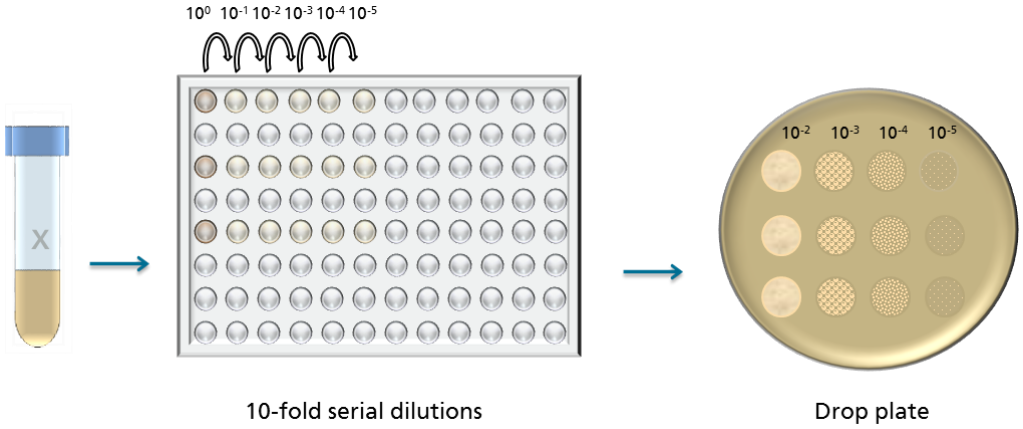


Fig. 21b. A scheme of the inhibition assay and the drop plate method.

3.7.1.1. *Influence of p2IgY and TIgY on the growth of TEM-1 producing E.coli*

E.coli were cultivated as described in subchapter 3.2.1. The bacteria cell suspension was adjusted to OD_{600} ~0.05 corresponding to a cell density of~2 × 10^6 cfu/ml. Specific IgYs solutions: p2IgY and TIgY were diluted with LB medium containing 0,1mg/ml ampicillin, to 10mg/ml. As a negative control a non-specific 10 mg/ml r^{I}IgY solution was used, and LB medium with 0.1mg/ml ampicillin without IgY was used as a blank. All the IgY solutions were mechanically sterilized using 0.22-µm membrane syringe filters. Each IgY solution and control were mixed 1:1 with a prepared *E.coli* suspension. Mixtures were incubated for 24h at 37˚C and 150rpm. At the time points 0h, 3h, 6h and 24h of incubation OD_{600} values of each mixture were measured, aliquots were taken and drop plates were prepared (n=6) in the way as described at the beginning of this chapter. Colony-forming units for each sample at all incubation time points were counted, total number of bacteria colony-forming units per ml for each incubation time point was calculated and plotted.

3.7.1.2. *Influence of ap2IgY and aTIgY on the growth of TEM-1 producing E.coli*

E.coli were cultivated as described in subchapter 3.2.1. The bacteria cell suspension was adjusted to OD_{600}~0.05 corresponding to a cell density of~2 × 10^6 cfu/ml. Specific IgYs solutions: ap2IgY and aTIgY were diluted with LB medium containing 0,1mg/ml ampicillin, to 0.2 mg/ml. As a negative control a non-specific 0.2mg/ml r^{IV}IgY solution was used, and LB medium with 0.1mg/ml ampicillin without IgY was used as a blank. All the IgY solutions were mechanically sterilized using 0.22-µm membrane syringe filters. Each IgY solution and control were mixed 1:1 (vol/vol) with prepared *E.coli*

suspension. Mixtures were incubated for 24h at 37˚C and 150rpm. At the time points 0h, 3h, 6h and 24h of incubation OD_{600} values of each mixture were measured, aliquots were taken and drop plates were prepared (n=3) in the way as described at the beginning of this chapter. Colony-forming units for each sample at all incubation time points were counted, total number of bacteria colony-forming units per ml for each incubation time point was calculated and plotted.

3.7.1.3. Influence of hIgY and eIgY on the growth of TEM-1 producing E.coli

E.coli were cultivated as described in subchapter 3.2.1. The bacteria cell suspension was adjusted to OD_{600} ~0.05 corresponding to a cell density of~2×10^6 cfu/ml. Specific IgYs solutions: h2IgY and eTIgY were diluted with LB medium without ampicillin, to 0.2 mg/ml. As a negative control a non-specific 0.2mg/ml r^{IV}IgY solution was used, and LB medium without ampicillin and without IgY was used as a blank. All IgY solutions were mechanically sterilized using 0.22-µm membrane syringe filters. Each IgY solution and control were mixed 1:1 (vol/vol) with prepared *E.coli* suspension. Mixtures were incubated for 24h at 37˚C and 150rpm. At the time points 0h, 3h, 6h and 24h of incubation OD_{600} values of each mixture were measured, aliquots were taken and drop plates were prepared (n=3) in the way as described at the beginning of this chapter. Colony-forming units for each sample at all incubation time points were counted, total number of bacteria colony-forming units per ml for each incubation time point was calculated and plotted.

3.7.2. Immunofluorescence staining

3.7.2.1. Specific binding of hIgY and eIgY to surface of TEM-1producing E.coli

E.coli were cultivated as described in subchapter 3.2.1 until OD_{600} reached 0.36. Cells were harvested by centrifugation at 4000g for 10min at 4˚C, washed twice with PBS and incubated in 4% formaldehyde in PBS, at RT for 20min. Subsequently, the cells were washed three times and resuspended in PBS. Fixed *E.coli* cells were incubated with primary antibodies diluted in 1xPBS containing 1% (w/vol) BSA (A9418, Sigma-Aldrich Chemie, GmbH, Taufkirchen, Germany) for two different time periods: one hour at 4˚C and overnight at 4˚C, with one of the three tested combinations: 1) eIgY + goat anti-*E.coli* IgG (OBT0986, Bio-Rad AbD Serotec GmbH, Puchheim, Germany), 2) hIgY + goat anti-E.coli IgG, 3) r^{III}IgY + anti-E.coli IgG. Then, samples were washed three times with PBS and incubated with secondary antibodies diluted in 1xPBS containing 1% (w/vol) BSA

in the dark for 1h at RT. Samples were washed three times with 1xPBS. Before applying them on a glass slides, the antifade reagent with DAPI was added (336939, Life Technologies Corporation). Visualization was performed on the Olympus IX81 inverted fluorescence microscope and using the CellF Software (Olympus, Aartselaar, Belgium). In the table 8 a detailed description of antibodies used in the staining is shown.

3.7.2.2. Specific binding of ap2IgY and aTIgY to TEM-1in E.coli culture

E.coli were cultivated as described in subchapter 3.2.1 until OD_{600} reached 0.37. Cells were harvested by centrifugation at 4000g for 10min at 4˚C, washed three times with PBS and incubated in 4% formaldehyde in 1xPBS in RT for 20min. Washed three times and resuspended in 1xPBS containing 1% (w/vol) BSA. Samples were permeabilized with 1xPBS containing 0.1% (vol/vol) Triton x-100 for 10min at RT, then washed with PBS and blocked with 1xPBS containing 2% (w/vol) BSA, for 1h at RT, and then washed again in 1xPBS. Then, the samples were incubated with primary antibodies diluted in 2% (w/vol) BSA with 1xPBS overnight at 4˚C in the one of the following four combinations: 1) aTIgY + goat anti-*E.coli* IgG, 2) ap2IgY + goat anti-E.coli IgG, 3) r^{III}IgY + goat anti-E.coli IgG, 4) ß-lac IgG1 (ab12251, Abcam plc, Cambridge, UK) + goat anti-E.coli IgG. Then samples were washed three times with PBS and incubated with secondary antibodies diluted in 1% (w/vol) BSA in PBS for 1h at RT, in the dark. Samples were washed three times with PBS and before applying them on the glass slides antifade reagent was added (336939, Life Technologies Corporation). Visualization was performed on the Olympus IX81 inverted fluorescence microscope and using the CellF Software (Olympus, Aartselaar, Belgium). In the table 9 a detailed description of antibodies used in the staining is shown.

3.8. Statistical analysis

All the data are presented as mean values ± SD. Statistical analysis for colorimetric detection of TEM-1 activity in *E.coli* culture was conducted using One-way ANOVA with Dunnett's multiple comparisons test. Statistical analysis of binding activity tests of IgYs via indirect ELISA were conducted using Two-way ANOVA with Bonferroni's multiple comparisons tests. Statistical analysis of inhibition assays were conducted using Two-way ANOVA with Tukey's multiple comparison tests.

Tab.8.

Primary antibody	Primary antibody type	Concentration	Secondary antibody	Dilution
eIgY	Polyclonal chicken IgY, anti-E.coli inactivated by e-beam 5kGy (Davids Biotechnologie)	50µg/ml	Rabbit Anti-chicken IgY FITC (ab6749, Abcam)	1:1000
hIgY	Polyclonal chicken IgY, anti-E.coli inactivated by heat (Davids, Biotechnologie)	50µg/ml		
r^{III}IgY	Polyclonal chicken IgY, non-specific (P075.05, Davids Biotechnologie)	50µg/ml		
anti-E.coli IgG	Polyclonal goat IgG, anti-E.coli (OBT0986, Bio-Rad AbD Serotec GmbH)	80µg/ml	Donkey Anti-goat IgG Alexa fluor 594 (705585147, Jackson Immunoresearch)	1:500

Tab.9.

Primary antibody	Primary antibody type	Concentration	Secondary antibody	Dilution
aTIgY	Polyclonal chicken IgY, anti-recombinant ß-lactamase precursor (Davids Biotechnologie)	20µg/ml	Rabbit Anti-chicken IgY FITC (ab6749, Abcam)	1:1000
ap2IgY	Polyclonal chicken IgY, anti-p2 (Davids, Biotechnologie)	20µg/ml		
r^{III}IgY	Polyclonal chicken IgY, non-specific (P075.05, Davids Biotechnologie)	20µg/ml		
anti-E.coli IgG	Polyclonal goat IgG, anti-E.coli (OBT0986 Bio-Rad AbD Serotec GmbH)	80µg/ml	Donkey Anti-goat IgG Alexa fluor 594 (705585147, Jackson Immunoresearch)	1:500
anti-ßlac IgG1	Monoclonal mouse IgG1, anti-ß-lactamase (ab12251, Abcam)	80ug/ml	Donkey Anti-mouse IgG Alexa Fluor 488 (A-21202, ThermoFisher Scientific)	1:200

Results and discussion

4. Results and discussion

4.1. Design of short peptides p1 and p2 for the development of IgYs against the active site of TEM-1

The structure of TEM-1, the firstly discovered ß-lactamase, is well studied and described. [33, 34] It is also classified as a parental enzyme for more than 200-member TEM group of ß-lactamases of which more than 86 were characterized as ESBLs. [36, 70, 81] To develop IgYs against TEM-1 active site, also with the perspective of targeting and inactivating TEM-1 mutants, the structure analysis was focused on the identification of low mutable amino acids residues playing important role in antibiotic hydrolysis process. Due to these requirements the sequences 1 and 2, containing these critical amino acids for TEM-1 activity, were chosen as a base for the p1IgY and p2IgY/ap2IgY development.

4.1.1. Catalytic sites and conservative residues in TEM-1 structure identified as a potential target for IgYs

Within the 263 amino acids of TEM-1, 7 amino acids are identified as important residues in catalytic site: S70, Lys73, Ser130, Lys234, Glu166, Asn170 and Ala237 (Fig.22). According to the literature, they have a specific role in the hydrolysis of ß-lactam antibiotics: Ser70 acts as a nucleophile in the attack on ß-lactam substrate carbonyl group, interacts with Glu166, stabilizes the tetrahedral intermediate during the process of acylation and deacylation and interacts with Lys73 by forming an ion pair together; Lys73 is involved in deprotonation of Ser70 actin as a general base in the acylation reaction and directs the hydroxyl group of S70 for the effective catalytic reaction, it also interacts with residue Glu166 through a hydrogen bond; Ser130 belongs to the hydrogen bonding network mediating protonation of the ß-lactam ring nitrogen and interacts with Lys234 and oxygen 12 from the carboxylate group of ß-lactam; Glu166 acts as a catalytic base towards the water molecule, what leads to deprotonation of Ser70 in the reaction of acylation and to the attack of the water molecule on the carbon of ß-lactam linked to the oxygen gamma of the residue S70 in the reaction of deacylation; Asn170 coordinates the water molecule by forming a hydrogen bond through its side chain and takes part in a proton transport from Ser70 to Lys73 in the acylation reaction; Lys234 recognizes ß-lactams at the initial step, electrostatically interacts with their carboxylate group and stabilizes transition states of the Michaelis-Menten complex, it also takes part in the protonation of ß-lactam nitrogen in the

acylation reaction and interacts with S130 via a hydrogen bond; Ala237 stabilizes a tetrahedral intermediate through the oxyanion hole which forms together with residue S70. [50, 54, 57, 60, 61, 74, 182, 188, 191–196]

The analysis with the Hot Spot Wizard shows that in the crystal structure of TEM-1 9 pockets can be found and the residues S70, S130, Lys234 and Ala237 all belong to the pocket 3 which is characterized as a catalytic pocket. The active site is situated between two domains of TEM-1 and consist of 4 important structural elements: 1) helix H2 which forms a bottom of an active site, the residues S70 and K73 are located here, 2) the SDN loop which is between helix H4 and helix 5, containing the residue S130, 3) the strand S5 with the residues Lys234 and Ala237, and 4) Ω-loop between amino acids 164-179 which forms a rim over the active site and contains residues Glu166 and Asn170. Additionally, the location of 7 residues in the different types of secondary structures was also analyzed: Ser70 and Asn170 are located on the 3(10) type of helix, Lys73 and Glu166 on the alpha helix, and Ser130, Lys234 and Ala237 on the hydrogen-bonded beta strands. [183, 197]

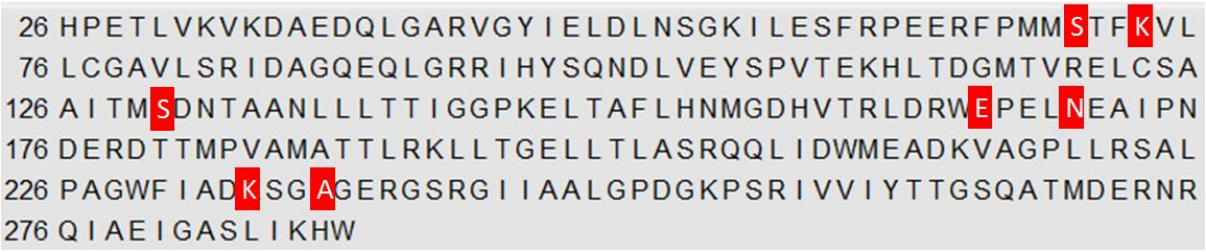


Fig. 22. The amino acid sequence of TEM-1 with 7 critical for the enzyme activity residues, highlighted in red: Ser70, Lys73, Ser130, Glu166, Asn170 , Lys 234 and Lys237.

Another important parameter taken into account was the mutability score of the 7 chosen catalytic residues in order to find the ones with the lower probability of mutations and therefore developing new TEM mutants. The analysis with Hot Spot Wizard program showed that 6 out of 7 residues were identified as conservative (low mutability): Ser70 (score 2), Lys73 (score 2), Ser130 (score 2), Glu166 (score 3), Asn170 (score 2), Lys234 (score 3), (fig. 23 and fig. 24).

Chain:	A
Position:	70
Amino acid:	Ser
Secondary structure:	3-10 helix (G)
Accessible surface area (ASA):	10.93 Å^2 (relative ASA: 9.0 %)
Mutability:	low
Mutability score:	2
Pockets:	3 (catalytic)
Tunnels:	1 starting from pocket 3.
Relative flexibility:	low
Average B-Factor:	20.59 Å^2

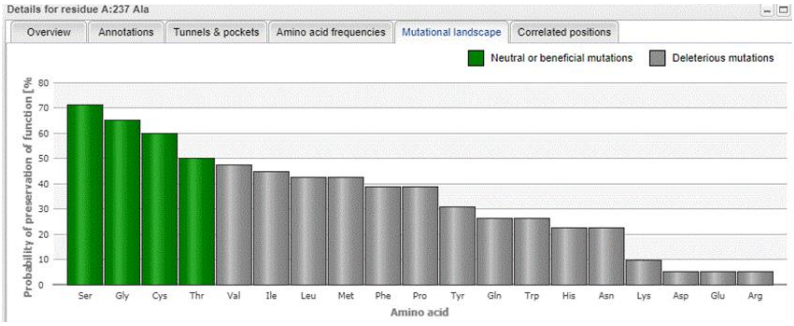
Chain:	A
Position:	73
Amino acid:	Lys
Secondary structure:	alpha helix (H)
Accessible surface area (ASA):	0.00 Å^2 (relative ASA: 0.0 %
Mutability:	low
Mutability score:	2
Pockets:	-
Tunnels:	1 starting from pocket 3.
Relative flexibility:	low
Average B-Factor:	17.55 Å^2

Chain:	A
Position:	130
Amino acid:	Ser
Secondary structure:	extended strand (E)
Accessible surface area (ASA):	12.84 Å^2 (relative ASA: 10.5 %)
Mutability:	low
Mutability score:	2
Pockets:	3 (catalytic)
Tunnels:	1 starting from pocket 3.
Relative flexibility:	moderate
Average B-Factor:	26.69 Å^2

Chain:	A
Position:	170
Amino acid:	Asn
Secondary structure:	3-10 helix (G)
Accessible surface area (ASA):	18.11 Å^2 (relative ASA: 11.5 %)
Mutability:	low
Mutability score:	2
Pockets:	-
Tunnels:	1 starting from pocket 3.
Relative flexibility:	moderate
Average B-Factor:	23.16 Å^2

Chain:	A
Position:	166
Amino acid:	Glu
Secondary structure:	alpha helix (H)
Accessible surface area (ASA):	5.15 Å^2 (relative ASA: 2.8 %)
Mutability:	low
Mutability score:	3
Pockets:	-
Tunnels:	1 starting from pocket 3.
Relative flexibility:	moderate
Average B-Factor:	28.13 Å^2

Chain:	A
Position:	234
Amino acid:	Lys
Secondary structure:	extended strand (E)
Accessible surface area (ASA):	0.32 Å^2 (relative ASA: 0.2 %)
Mutability:	low
Mutability score:	3
Pockets:	3 (catalytic)
Tunnels:	1 starting from pocket 3.
Relative flexibility:	low
Average B-Factor:	21.91 Å^2



Chain:	A
Position:	237
Amino acid:	Ala
Secondary structure:	extended strand (E)
Accessible surface area (ASA):	43.59 Å^2 (relative ASA: 38.6 %)
Mutability:	moderate
Mutability score:	5
Pockets:	3 (catalytic)
Tunnels:	1 starting from pocket 3.
Relative flexibility:	low
Average B-Factor:	22.35 Å^2

Fig. 23. Characteristics of Ser70, Lys73, Ser130, Glu166, Asn170 , Lys 234 and Lys237. Analysis done with the Hot Spot Wizard, revealed that the residue with the highest mutability score among the other was Ala237. Furthermore, it indicated that the possible exchange with amino acids: Ser, Gly, Cys or Thr might preserve the role of the residue in the active enzyme, or it can be even beneficial.

The mutability of the residue Ala237 was predicted at the level of 5 which is defined as moderate. When compared this with Lahey data base (https://www.lahey.org/Studies/), the mutability of these residues among 223 of TEM-1 derivatives was as follow: Ser130 was substituted by Gly residue in derivatives TEM-59 (gene bank accession AF062386), TEM-76 (AF190694), CMT-3 (AY039040) and by Thr in TEM-211 (KF513179); Glu166 residue was replaced by Gly in TEM-193 (JN935135); Asn170 no substitutions were reported; Ala237 substituted by Thr in derivatives: TEM-5 (no accession code), TEM-24 (X65253), TEM-86 (AJ277415), TEM-114 (AY589495), TEM-121 (AY271264), TEM-130 (AJ866988), TEM-131 (AY436361), TEM-136 (AY826417), TEM-177 (FN652295) and by Gly in TEM-22 (Y17583). For the residues: Ser70 and Lys73 data were not shown, it might be due to their critical role and presence in the whole class A of ß-lactamases; for the Lys234 data were also not shown.

All these data were complemented with the research of Brown et al. 2009. They aligned the amino acid sequences of the Ω-loop of different representatives of ß-lactamases types from class A and it was established that more than 96% of ß-lactamases class A had the residue Asn at the position 170 and all of them had the Glu at the position 166.

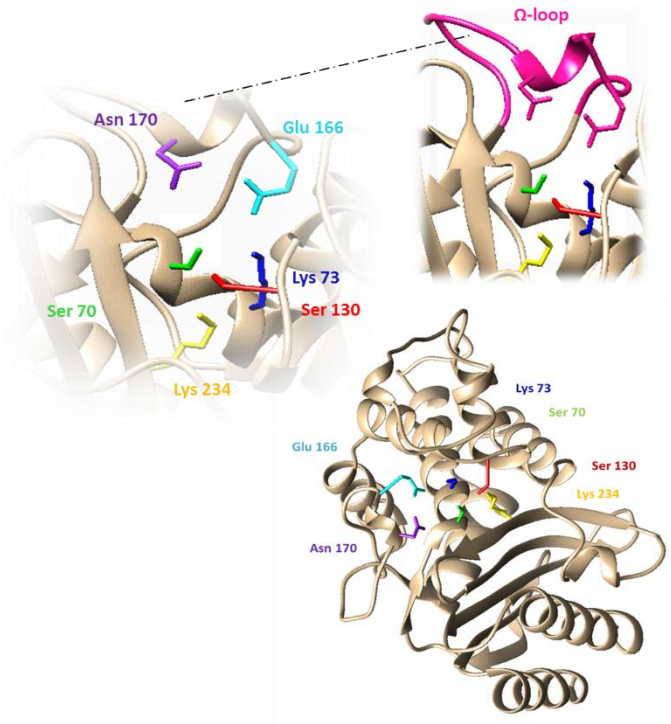


Fig. 24. The 3D structure of TEM-1 with 6 conservative catalytic residues highlighted in different colors: Ser70 in light green, Lys73 in dark blue, Ser130 in red, Glu166 in torques, Asn170 in violet and Lys234 in yellow. The Ω-loop including the Glu166 and the Asn170 is highlighted in pink.

Based on the summary of this data analysis, the following optimal candidate target residues for the IgY development against the active site of TEM-1 were chosen: Ser70, Lys73, Glu166 and Asn170. All of them show low mutability tendency and they play a crucial role in ß-lactams hydrolysis. Moreover, they are situated in strategically good locations in TEM-1 structure (bottom of the active site and Ω-loop above the active site) and they could be paired, what was convenient for choosing the sequences for short peptides synthesis mimicking the active site.

4.1.2. Short peptides p1 and p2 mimic the functionally important structures of TEM-1 active site

Two structures of the TEM-1 active site were chosen to be targeted by IgYs as the base for one of the strategies against *E.coli* producing TEM-1: the H2 helix and the Ω-loop. The length of sequences was 11 amino acids and they included previously identified and selected catalytic residues. Sequence 1 (RFPMMSTFKVL) is placed between amino acids 65-75 from the H2 helix and includes residues Ser70 and Lys73, sequence 2 (TRLDRWEPELN) is located between amino acids 160-170 from the Ω-loop and includes residues Glu166 and Asn170 (fig. 25 a and fig. 26 a).

The choice of the length of the sequences was dictated by general requirements for the generation of IgYs. Sequences of 10-20 amino acids minimalize technical problems with the peptide synthesis. While anti-short-peptides antibodies are very specific to antigen, those based on sequences longer than 10 amino acids have higher chances to recognize the native structure of protein. Though, longer sequences increase the recognition of bigger number of epitopes. There are several studies on specific IgYs against pathogens, more specifically, IgYs developed against virulent protein fragments or linear short peptides mimicking those, where their therapeutical properties were presented. Moreover, Shin et al. 2003 reported effectivity of IgYs against H. pylori that were developed against a 15 amino-acids-short peptide mimicking Urease B, Schade et al. 1996 demonstrated the development of specific IgYs against 8 amino-acids cholecystokinin octapeptide for immunohistochemistry assays. [198–201] Based on these studies the length of 11 amino acids was chosen as the optimal.

A very important parameter in generating specific antibodies is the antigenicity of the synthesized short peptides. It should be high enough to boost the immune response in immunized chickens in order to produce the required IgYs. To predict the antigenicity of short peptides p1 and p2, which correspond to chosen sequences 1 and 2 respectively, the algorithm-based Antigen Profiler Peptide Tool - provided online by Thermofisher- was used. For the short peptides p1 and p2, the score for theoretical antigenicity was 3 and 4.1 respectively, what indicated their properties as ‘excellent antigens’ (fig. 25 b and fig. 26 b). Additionally, a 3D visualization of the sequence 1 and the sequence 2 location in TEM-1 structure was done (fig. 25 c and fig. 26 c). It was necessary to show whether the developed p1IgYs (IgY against sequence 1) and p2IgYs (IgY against sequence 2) can potentially target these sequences in the 3D conformation of TEM-1. As it can be seen in fig. 27, the sequence 2 in the Ω-loop is more exposed than the hidden sequence 1, which potentially makes it an easier target.

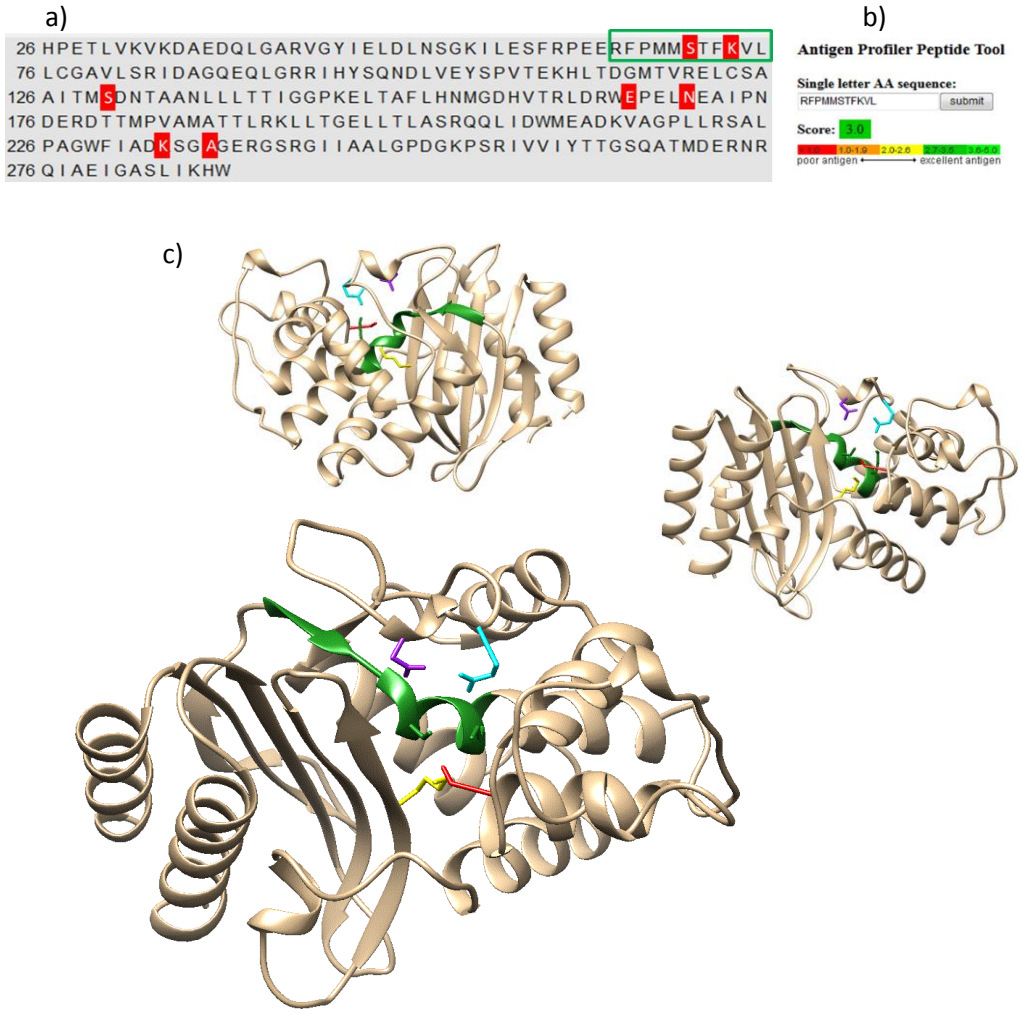


Fig. 25. Sequence 1 in TEM-1. a) The chosen sequence was: RFPMMSTFKVL and contained Ser70 and Lys73. b) The peptide based on this sequence was recognized as an excellent antigen by Antigen Profiler Peptide Tool. c) The location of sequence 1 in 3D structure of TEM-1.

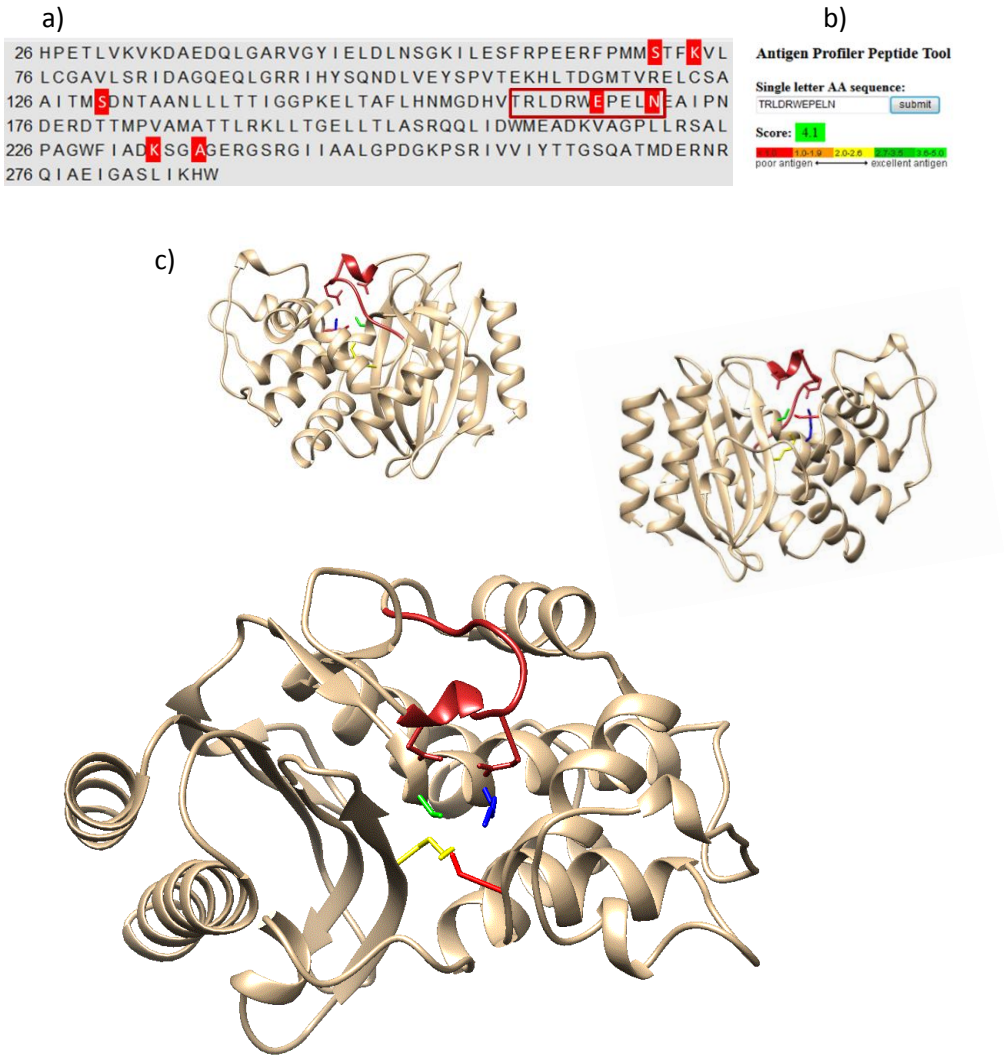


Fig. 26. Sequence 2 in TEM-1. a) The chosen sequence was: TRLDRWEPELN located in the Ω-loop and contained Glu166 and Asn170. b) The peptide based on this sequence was recognized as an excellent antigen by Antigen Profiler Peptide Tool. c) The location of sequence 2 in 3D structure of TEM-1.

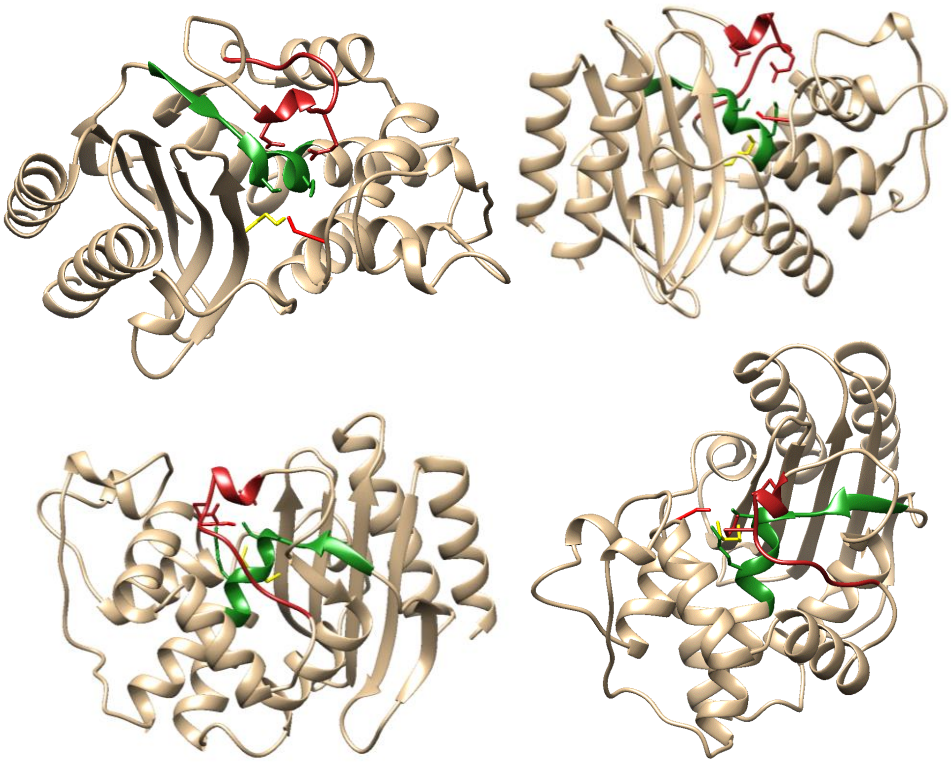


Fig. 27. The location of sequence 1 (green) and sequence 2 (red) in 3D structure of TEM-1.

4.2. IgYs against ß-lactamase TEM-1 used as complement to antibiotic treatment reduced the growth of TEM-1- producing *E.coli in vitro*

Previous studies showed that it is possible to inhibit the growth of bacteria by a treatment IgYs developed against toxins, subunits of toxins or recombinant protein structures mimicking virulent factors from the bacteria cell surface. [149, 150, 202, 203] Here, IgYs were developed against the whole ß-lactamase TEM-1 and the two structures within the active site, in order to inactivate the enzyme and allow antibiotic to eradicate the bacteria. The assumption of this set up was that blocking TEM-1 by IgYs will help ampicillin to overtake the *E.coli* resistance and kill the bacteria.

To confirm the secretion and activity of TEM-1 by the *E.coli* used in this study, a control experiment was done using nitrocefin. This is a chromogenic cephalosporin sensitive for ß-lactamase activity. Due to the ß-lactam ring it possesses, it gets hydrolyzed in the presence of ß-lactamase and changes color from yellow to red. Out of the four tested samples, two showed the color change: cell lysate (CL) of TEM-1 producing *E.coli* changed the color from yellow to dark red, and *E.coli* cell culture (CC) to orange (Fig. 28, 2)). Samples of LB medium and PBS were used as controls and did not show any color changes. It means that TEM-1 activity was detected only in *E.coli* cell culture and *E.coli* cell

lysate. Additionally, the darker red color in the cell lysate which was correlated with a higher activity of the ß-lactamase, might confirm that TEM-1 is likely secreted into periplasmic space rather than extracellularly. [204] The samples were also tested spectrophotometrically (fig. 28, 1), where the values of absorption at 486nm for samples with *E.coli* cell lysate and *E.coli* cell culture were significantly higher when compared to control samples (PBS and LB medium). The absorption in the samples with *E.coli* cell lysate were significantly higher than for the *E.coli* cell culture ($p<0.05$).

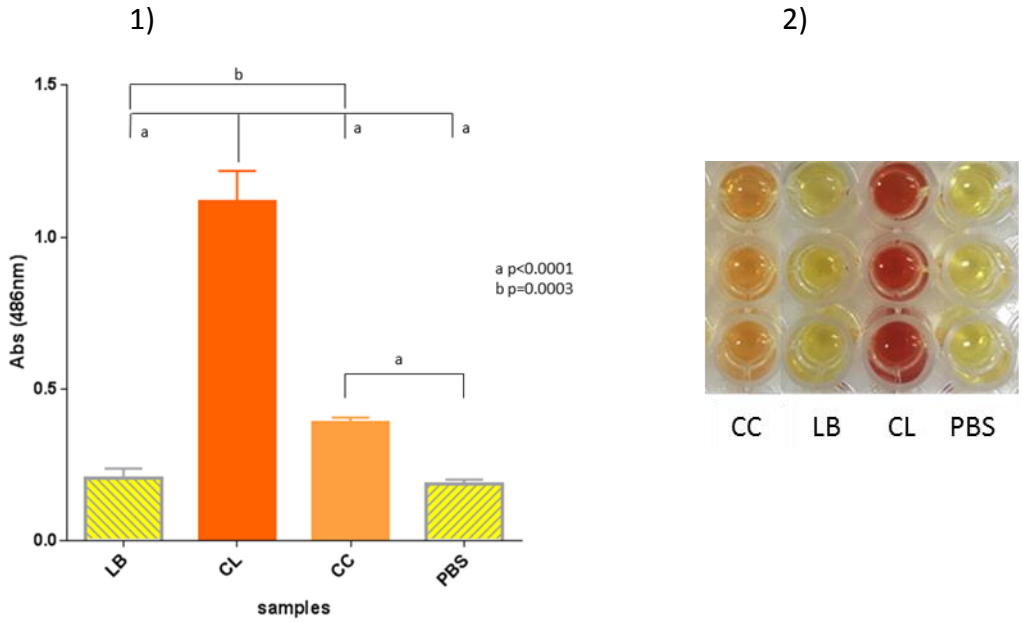
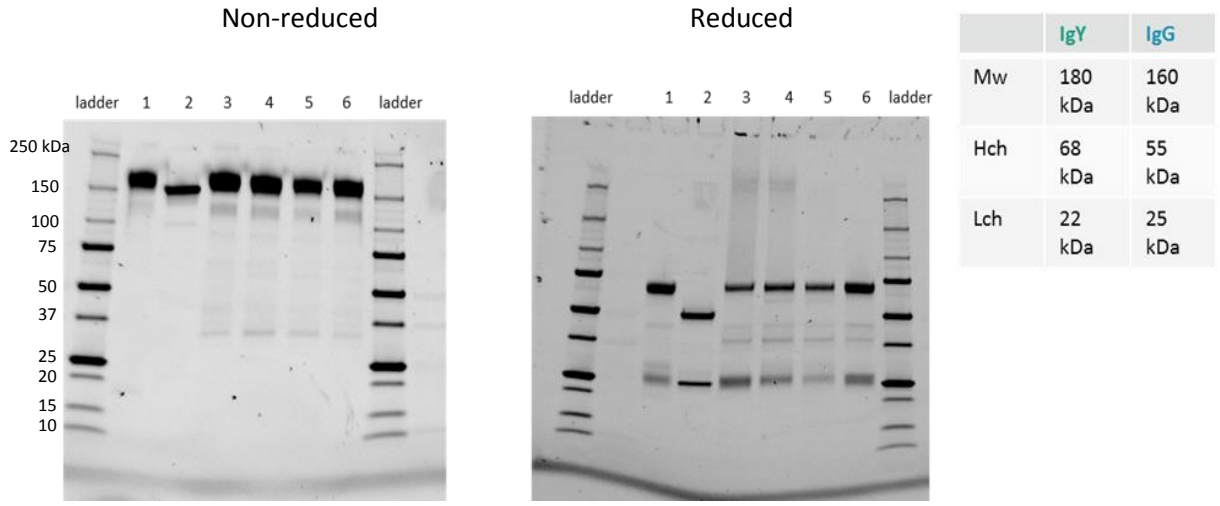


Fig. 28. Colorimetric detection of TEM-1 activity in *E.coli* cell culture (CC) and cell lysate (CL) in the presence of nitrocefin: 1) spectrophotometrically, 2) visually. *E.coli* were first cultivated in LB medium, then half of the samples were sonicated in order to obtain cell lysate. The other half was left untreated as samples of the cell culture. PBS and LB medium were used as controls. (LB n=6, CL n=3, CC=3, PBS n=6, p-values of less than 0.05 were considered as statistically significant)

4.2.1. Characteristics of specific IgYs purified by precipitation and their binding activity against TEM-1

Even though the function of IgYs and IgGs is similar, due to their phylogenetical distance, they show many other differences (described in chapter 1.10). Structural difference can be clearly seen with SDS-page electrophoresis when some of the IgYs developed in this study are compared to IgG (fig. 29).

In this method, the sodium dodecyl sulfate (SDS) imparts a negative charge to all the proteins and their separation is only due to differences in molecular weight.



	IgY	IgG
Mw	180 kDa	160 kDa
Hch	68 kDa	55 kDa
Lch	22 kDa	25 kDa

Fig. 29. Comparison of the structure bewteen IgYs and IgG by SDS-page electrophoresis in non-reduced and reduced conditions: 1- p1IgY, 2- IgG, 3- r^{II}IgY, 4- TIgY, 5- r^{I}IgY, 6- r^{III}IgY. Before loading on the gel, antibodies samples were mixed with Laemmli buffer, for the reduced condition additionally with ß-mercaptoethanol, and incubated at 95˚C for 5min. Separation of immunoglobulins and their elements in both set-ups was due to differences in molecular weight. The table shows the differences in molecular weight (Mw) of IgYs and IgG, their heavy chains (Hch) and light chains (Lch).

In the non-reduced condition, clear black bands can be seen at the level of 180kDa for samples containing IgYs: p1IgY (IgY against peptide 1), r^{I}IgY, r^{II}IgY, r^{III}IgY (non-specific IgYs), TIgY (IgY against TEM-1), and at the level of 160kDa for IgG. In the reduced condition, the presence of ß-mercaptoethanol disrupts the disulfide bonds between protein structures. Consequently, immunoglobulins were separated into structures of heavy and light chains. For IgYs, clear black bands could be seen at the level of 68kDa in the lanes 1,3,4,5 and 6, and for IgG at the level of 55kDa in the lane 2, indicating the presence of the heavy chains of each immunoglobulin type. Regarding the IgG light chains, a clear band was observed at the 25kDa. For IgYs, bands are smeared, but their bottom start below the IgG band, at around 22kDa and go above 25kDa.

Results can be compared to the ones produced by Nasiri et al. and Amro et al. [205, 206] Appearance of additional IgY bands or bands which are a bit smeared, in both reduced and non-reduced SDS-page, might be due to the fact that IgG used in this experiment is a monoclonal affinity

purified antibody type, whereas IgYs p1IgY, r^{I}IgY, r^{II}IgY, r^{III}IgY, TIgY were not affinity purified and solution might have contained impurities from the egg yolk, other protein structures or not well developed IgYs. [206] For instance, bands around 40kDa might indicate the presence of C-terminal fragment of the vitellogenin II precursor.[207]

At the first step of this study, IgYs against TEM-1 beta lactamase p1IgY, p2IgY and TIgY were purified by precipitation, which is the fastest and cheapest method for obtaining high amounts of IgYs.[116] The developed IgYs were analyzed via indirect ELISA using a twofold dilution series starting from a concentration of 200 µg/ml, firstly, to test their binding activity against the Beta lactamase TEM precursor protein (purified TEM-1) in order to choose the best binders, and, secondly, to test their activity towards TEM-1-producing *E.coli* lysate.

At concentrations of 200-50 µg/ml, the ELISA results revealed a better binding ability of p2IgY and TIgY to purified TEM-1 compared to p1IgY and rIgY (a negative control)($p < 0.05$) (fig. 30, a). Below these concentrations, only TIgY showed the best binding capacity to purified TEM-1 amongst all tested antibody types. TIgY gave a signal even at a concentration as low as 1,56µg/ml. p1IgY was not able to bind to TEM-1. Based on this observation, p1IgY was excluded from further experiments. The reason why p1IgY did not bind to TEM-1 might be the location of sequence 1. It could be that the steric hindrances of the region around H2 helix made the location of the sequence 1 inaccessible for the specific p1IgY. Hence, p1IgY would not be able to reach the active site and potentially inactivate it. No non-specific binding of rIgY was observed.

The results of the TEM-1-producing *E.coli* cell lysate ELISA showed that TIgY had the highest binding activity to TEM-1 present in the lysate (fig. 30, b). It was detected starting form the highest concentration of 200µg/ml until the concentration 6.25µg/ml ($p<0.05$). The reason why p2IgY binding was not detected in this set up might be that the concentration of TEM-1 secreted by *E.coli* is much lower than the concentration of purified TEM-1 applied in the previous setting (1µg/ml). No significant difference was showed between values for p2IgY and rIgY.

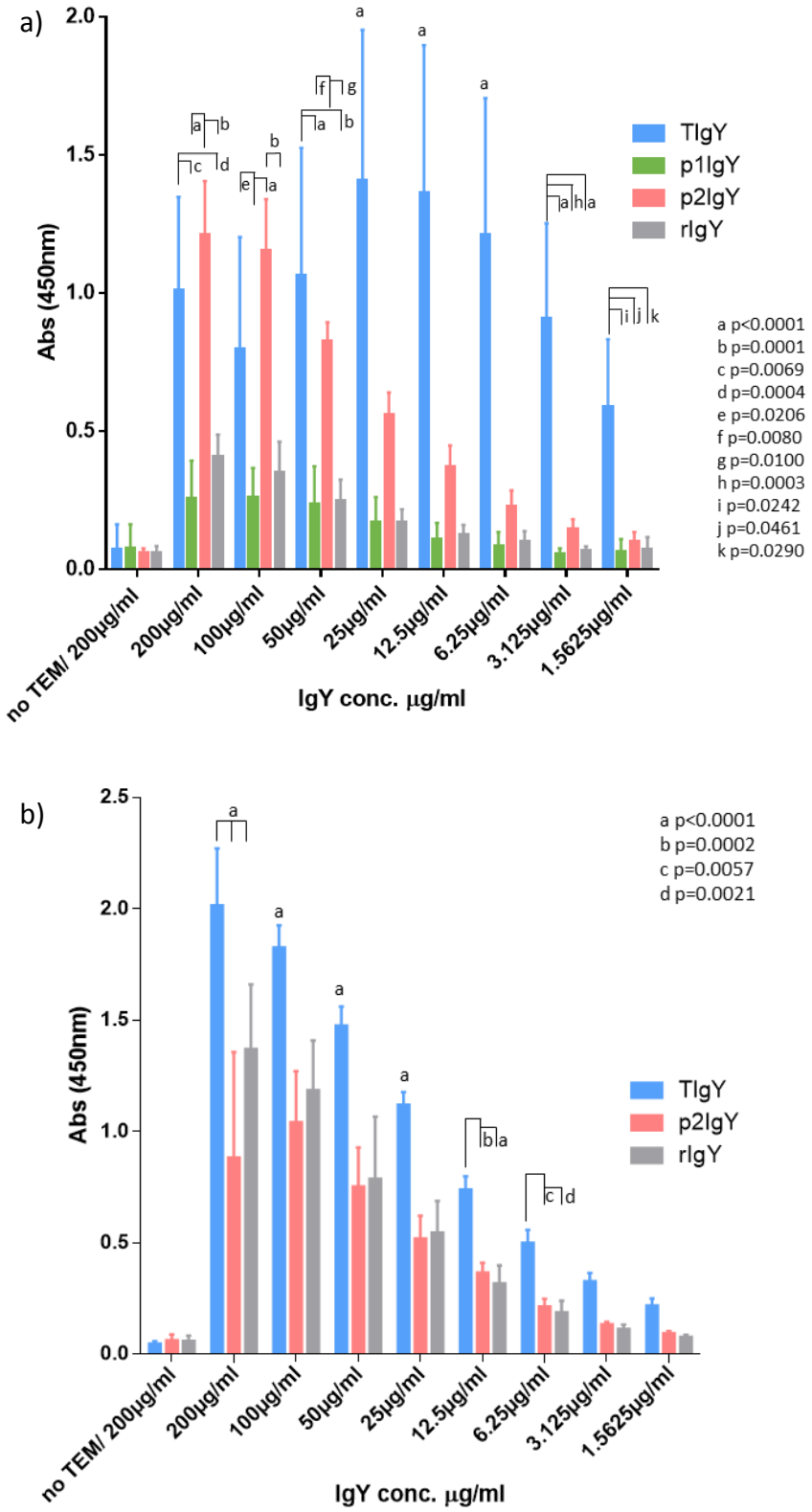


Fig.30. Comparison of binding activity between precipitation - purified IgYs against: a) TEM-1 (n=3, performed in triplicates) and b) the lysate of TEM-1-producing *E.coli* (n=2 performed in triplicates). In the first set-up a 96 well plate was coated with 1µg/ml of the Beta lactamase TEM precursor protein. In the second set-up the plate was coated with the lysate of TEM-1-producing *E.coli* obtained by sonication, in the concentration corresponding to ~1.2x106 CFU/ml. As the primary antibodies p1IgY (precipitation-purified IgY against peptide 1), p2IgY (precipitation-purified IgY against peptide 2), TIgY (precipitation-purified IgY against TEM-1) and rIgY (non-specific IgY) were used. Secondary anti-IgY antibodies were conjugated to a horse radish peroxidase for the binding visualization. (p-values of less than 0.05 were considered as statistically significant)

4.2.2. *In vitro* growth inhibition of TEM-producing *E.coli* by TEM-1-specific and precipitation-purified IgYs in the presence of ampicillin

The inhibition assay where a TEM-1-producing *E.coli* liquid cell culture was incubated for 24h with 5mg/ml of p2IgY, TIgY and rIgY, in LB medium in the presence of ampicillin revealed that all of IgYs showed an inhibitory effect on the growth of TEM-1-producing *E.coli*. Similar results were obtained by Sugita et al., Tobias et al. and Shin et al., where specific IgYs purified by the precipitation method were used *in vitro* in a range of 1-10mg/ml against different pathogens and a decreased growth of bacteria was observed even after the treatment with unspecific IgYs. [147, 152, 157, 202] It might be due to the purification method: in a non-affinity purified population of IgYs, even up to ~98% of IgYs might be unspecific and it can increase over 99% when the chicken was immunized with a short peptide conjugated to KLH (according to the information of manufacturer http://gallusimmunotech.com/custom-services/affinity-purification).

The OD_{600} measurements of *E.coli* liquid cultures with different treatments in the presence of ampicillin showed a significant decrease of bacteria cell density when incubated with TIgY, p2IgY and, interestingly, with rIgY, in comparison to sample without the treatment - LB medium + ampicillin ($p<0.05$)(fig.31, a). Moreover, the inhibitory effect of TIgY and rIgY was greater than the one of p2IgY after 24h of incubation and the decrease in cell density was significantly higher ($p<0.05$). At every specified time point of incubation (0h, 3, 6h and 24h), samples from each condition were drop-plated in serial dilutions in order to count the number of living bacteria as a colony forming unit. The CFU/ml in each liquid culture samples was calculated after the treatment with TEM-1-specific IgYs and controls. A representative example of drop plates established in this inhibition assay is showed on fig. 31, b. Results revealed that the treatment with p2IgY, TIgY, rIgY in the presence of ampicillin inhibited significantly the growth of TEM-1 producing *E.coli* when compared to sample without any treatment (LB + ampicillin) ($p<0.05$) (fig.32, a).

Data were also plotted as logarithmic values to present how each of the treatments influenced the grow curves of TEM-1-producing *E.coli* (fig.32, b). It can be observed that the grow curve of *E.coli* incubated with TIgY dropped significantly in comparison to the rest of the samples. Also the treatment with rIgY decreased the growth of bacteria significantly. The result for p2IgY shows that the growth of *E.coli* was also inhibited but not as strongly as in samples treated with either TIgY or rIgY.

These results might be falsely influenced though by the very high concentration of IgYs used in this study, and two explanatory hypothesis might be correlated with it: 1) when IgYs are purified with the precipitation method, the amount of highly specific antibodies is usually at the level of ~2-16% of total IgYs what might have caused unspecific binding in the *in vitro* studies, especially, when the high concentration of antibodies were used, 2) to prepare such a high concentration of IgYs, more volume of original IgYs solution was used, contained up to 0.075% sodium azide, which might show toxic

effects towards *E.coli* cells. Consequently, this could have negatively influenced the growth of the bacteria. No toxicity test with IgY buffer were done in this study.

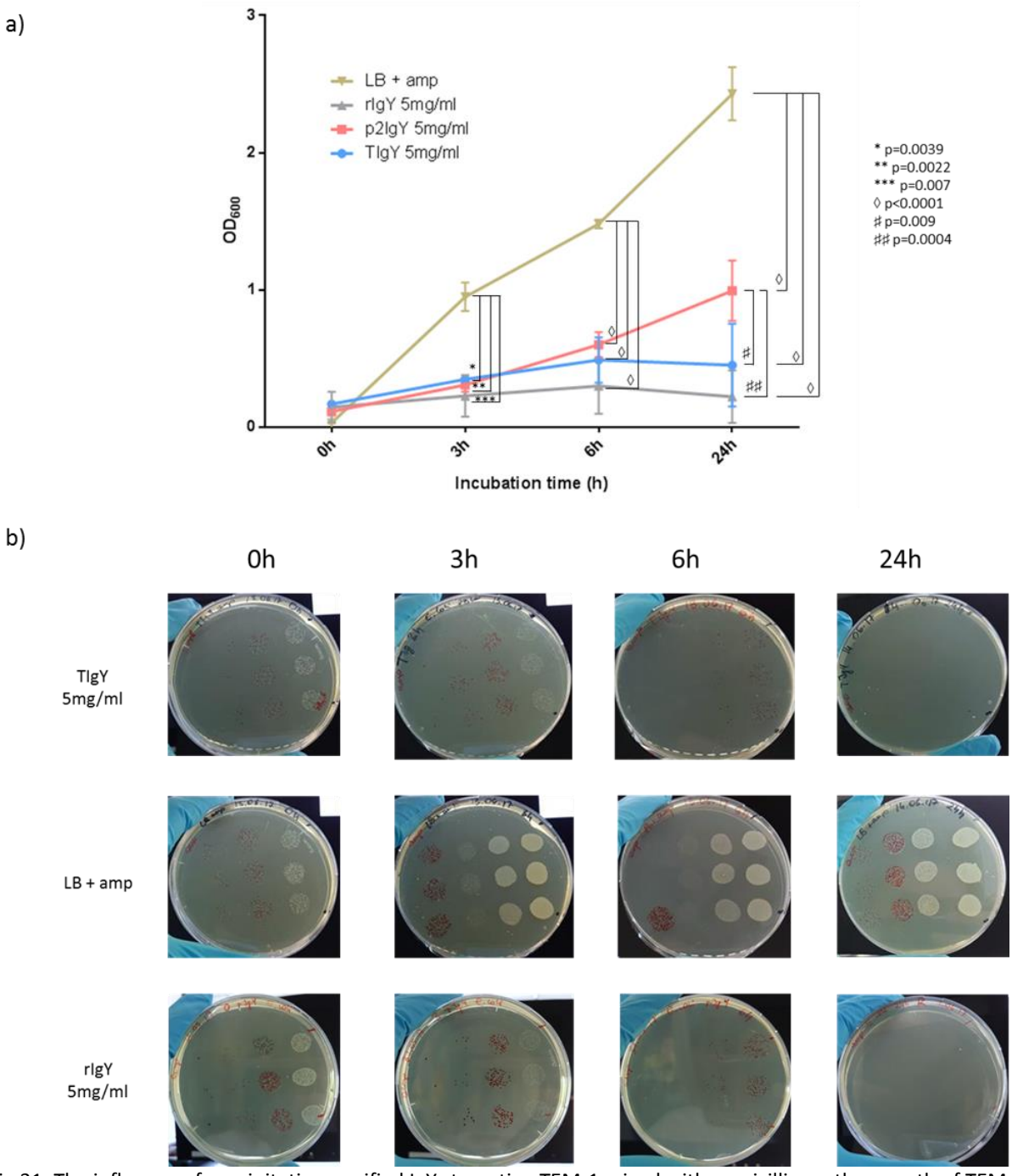


Fig.31. The influence of precipitation-purified IgYs targeting TEM-1 mixed with ampicillin on the growth of TEM-1-producing *E.coli*. a) The optical density measurement, (n=2, p-values of less than 0.05 were considered as statistically significant), b) the representative example of drop plates used for the calculation of colony forming unit. *E.coli* inoculates were incubated for 24h in the presence of 5mg/ml p2IgY and TIgY. As controls LB medium with ampicillin (no IgY) and non-specific IgYs were used (rIgY). To compare the growth of *E.coli* after incubation with different IgYs by the drop plate method, samples were taken at the time points: 0h, 3h, 6h and 24h.

Fig.32. Inhibitory effect of precipitation-purified IgYs targeting TEM-1 mixed with ampicillin, on TEM-1-producing *E.coli*: a) influence of different treatments on number of colony forming units, b) influence on growth curve. (n=6, p-values of less than 0.05 were considered as statistically significant)

4.2.3. Binding activity of affinity purified IgYs against TEM-1

In order to increase the specificity of IgYs to the TEM-1 ß-lactamase and, therefore, decrease the high unspecific binding noticed in previous set-up, IgYs targeting the sequence 2 of the TEM-1 active site and targeting the whole enzyme TEM-1 were affinity purified, named ap2IgY and aTIgY, respectively. In a two-fold dilution series ELISA on the lysate of TEM-1-producing *E.coli*, starting from 80µg/ml as the highest concentration, the results revealed that among ap2IgY, rIgY and aTIgY, the latter showed the highest binding activity to TEM-1, ($p<0.05$) (fig.33.). Interestingly, these results show a similar tendency as precipitation purified TIgY. Although, it can be appreciated that due to the affinity purification the specificity increased. The aTIgY still showed binding specificity at a concentration of 2.5 µg/ml, while the TIgY showed binding signal at the minimal concentration of 6.25 µg/ml.

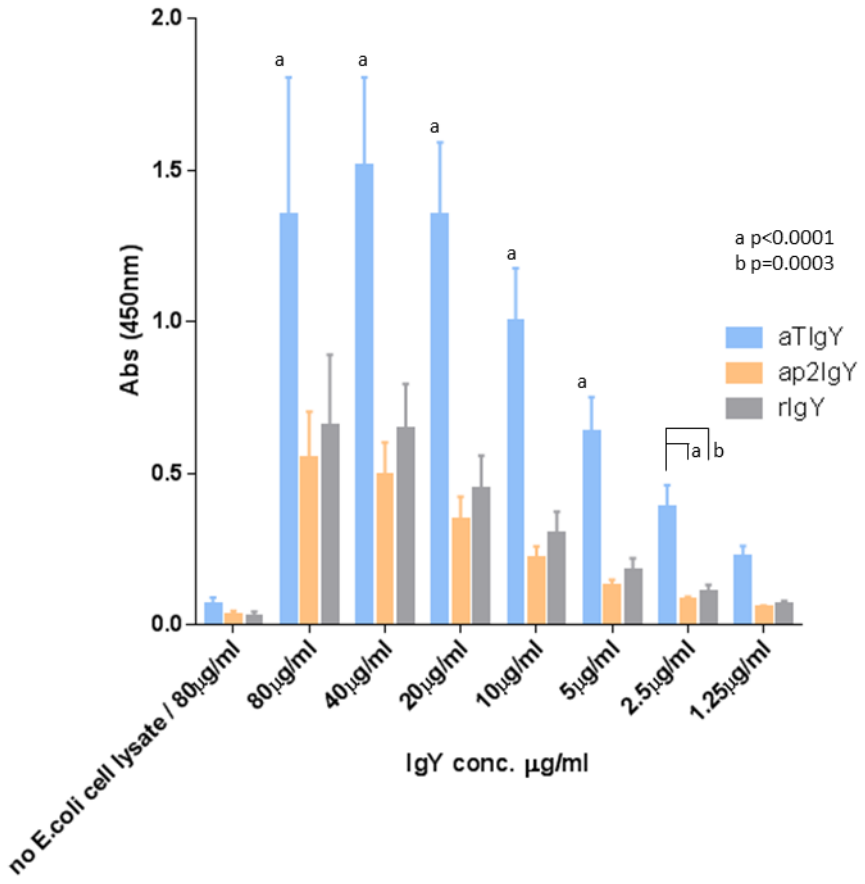


Fig.33. Comparison of binding activity between affinity-purified IgYs targeting TEM-1 against the lysate of TEM-1-producing *E.coli*. The 96 well plate was coated with the lysate of TEM-1-producing E.coli obtained by sonication, in the concentration corresponding to ~1.2x106 CFU/ml. As the primary antibodies ap2IgY (affinity-purified IgY against peptide 2), aTIgY (affinity-purified IgY against TEM-1) and rIgY (non-specific IgY) were used. Secondary anti-IgY antibodies were conjugated to a horse radish peroxidase for the binding visualization. (n=3 performed in triplicates, p-values of less than 0.05 were considered as statistically significant)

4.2.4. In vitro growth inhibition of TEM-1-producing *E.coli* by affinity purified IgYs specific to TEM-1 in the presence of ampicillin

To test the influence of affinity-purified IgYs as a compound complementary to antibiotic on the growth of TEM-1-producing *E.coli in vitro*, an inhibition assay was performed by incubating TEM-1-producing *E.coli* liquid cell culture for 24h with 0.1/ml of ap2IgY, aTIgY and rIgY in LB medium in the presence of ampicillin. It revealed that ap2IgY and aTIgY inhibited the growth of TEM-1-producing *E.coli* in culture. In fig.34 a pictures of the TEM-1-producing *E.coli* cell culture samples during the incubation with different IgYs after 6h and 24h are shown. Here, the color and transparency differences between the samples are noticeable: the appearance of the ap2IgY- and aTIgY-incubated cultures at the time point 6h and 24h of incubation were transparent, but the color was more intense and less transparent at 6h, even completely turbid at 24h when the cultures were incubated with rIgY or LB alone.

These results correlated with the OD_{600} measurements, where a dramatical decrease of the *E.coli* cell density was observed after the ap2IgY and aTIgY treatment, while rIgY treatment had no effect on *E.coli* growth (fig.34 b). Importantly, this experiment requires more repeats for a reliable interpretation.

Fig. 34 c shows a representative example of drop plates established in this inhibition assay. Results revealed that the treatment with ap2IgY and TIgY in the presence of ampicillin significantly decreased the number of CFU/ml in treated samples of TEM-1-producing *E.coli* cultures in comparison to rIgY and LB ($p<0.05$)(fig. 35 a). In this study, rIgY at the concentration of 0,1mg/ml did not show unspecific inhibitory effect. The logarithmic values showed that the growth curves of TEM-1-producing *E.coli* treated with ap2IgY and aTIgY had a decreasing tendency and were significantly different from those representing treatment with rIgY or LB alone ($p<0.05$)(fig. 35 b).

These results showed that affinity purified ap2 and aTIgY developed against TEM-1, at low concentrations such as 0,1mg/ml and in the presence of ampicillin show inhibitory effect against TEM-1 producing *E.coli* strain *in vitro*. It might be that ap2IgY and aTIgY target the active site and coat the whole enzyme, respectively, and as a consequence inactivate the TEM-1 so the ampicillin can eradicate the *E.coli*. However, the molecular details of this machinery have not been still investigated. Nevertheless, there are studies confirming neutralizing properties of specific IgYs against virulent proteins secreted by pathogens, e.g. Shiga toxin 2e produced by *E.coli* or Neurotoxin A produced by *C.botulinum.*[150, 203] Additionally, Shin et al. reported development of an effective IgY inactivating a secreted-by-*H.pylori* Urease B, based on *de novo* synthesised short peptide representing the enzyme.[199] No studies regarding development of IgYs to neutralise ß-lactamase was found though.

Immunofluorescent staining microscopy photographs visualized the binding specificity of ap2IgY and aTIgY in comparison to commercially available monoclonal IgG1 against ß-lactamase, which was treated as a positive control and rIgY as a negative control.

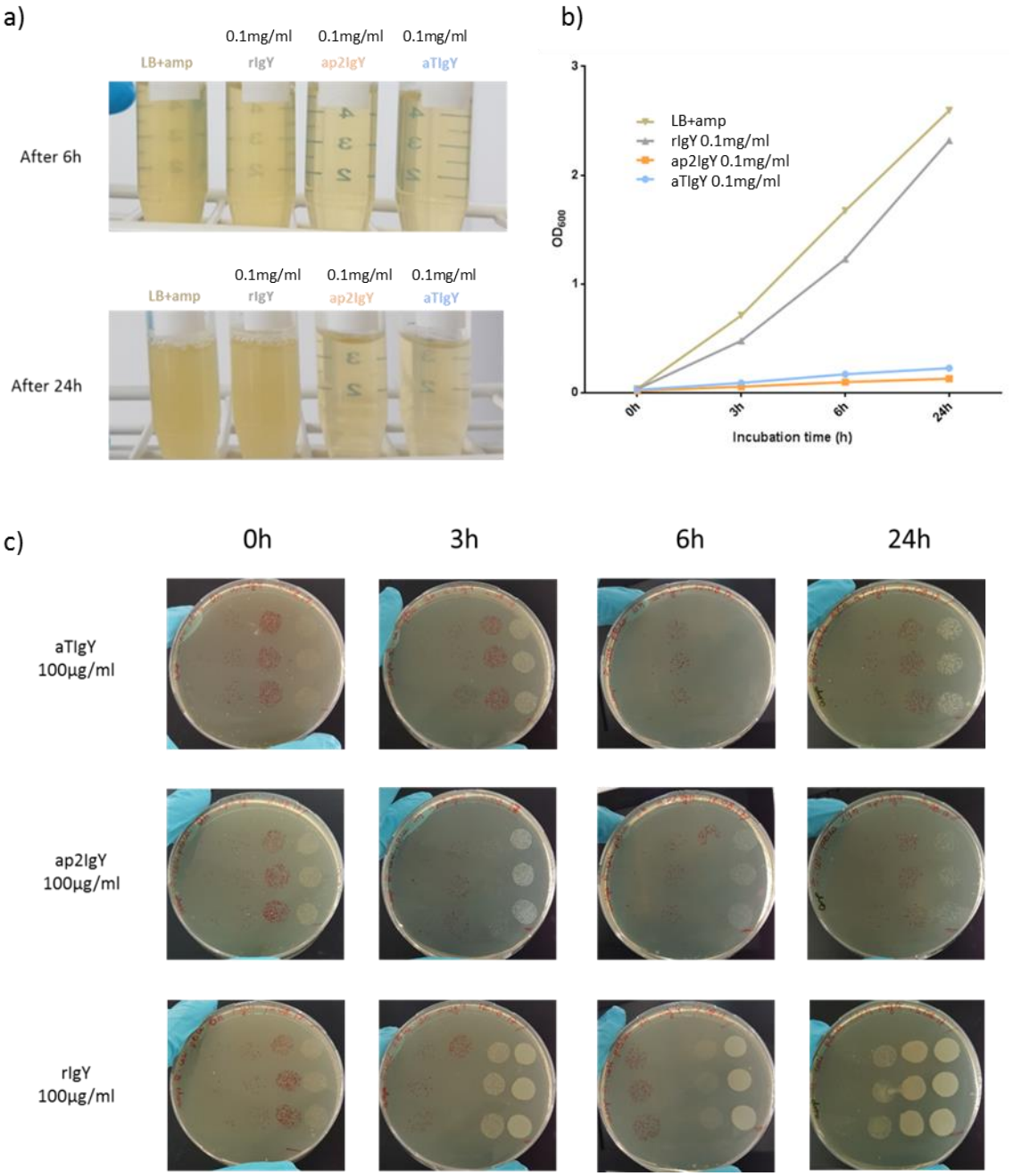


Fig.34. The influence of affinity-purified IgYs targeting TEM-1 mixed with ampicillin on the growth of TEM-1-producing *E.coli*. a) The visual changes in samples after 6 and 24h of incubation, b) optical density measurement, (n=1), c) the representative example of drop plates used for the calculation of colony forming unit. *E.coli* inoculates were incubated for 24h in the presence of 100µg/ml ap2IgY and aTIgY. As controls LB medium with ampicillin (no IgY) and non-specific IgYs were used (rIgY). To compare the growth of *E.coli* after incubation with different IgYs by the drop plate method, sam at the time points: 0h, 3h, 6h and 24h.

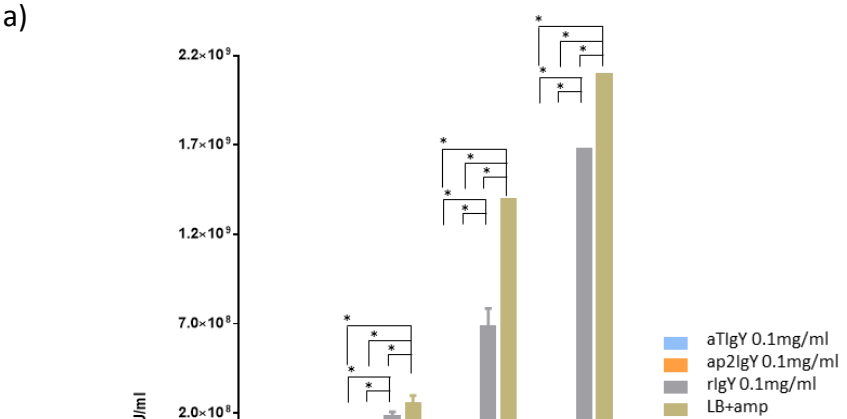


Fig.35. Inhibitory effect of affinity-purified IgYs targeting TEM-1 mixed with ampicillin, on TEM-1-producing *E.coli*: a) influence on number of colony forming units, b) influence on growth curve. (n=3, p-values of less than 0.05 were considered as statistically significant)

4.2.5. Immunofluorescent staining of TEM-1 in *E.coli* using affinity purified IgYs

Immunofluorescence staining was employed to visualize the binding specificity of ap2IgY and aTIgY to TEM-1 in formaldehyde-fixed *E.coli* (fig.36-38). Commercially available monoclonal IgG1 against ß-lactamase was used as a positive control. Additionally, the *E.coli*-specific IgG was used to stain the surface of bacteria cells and rIgY as a negative control. Whereas *E.coli*-specific IgG stained the surface of all cells, ß-lactamase-IgG1 gave a signal in the periplasmatic space (fig. 36, upper picture).

The staining pattern of ß-lactamase-IgG1 observed here reproduces the observations of Rohde et al. [208] The negative control rIgY did not show any signal (fig. 36, bottom picture). Interesting observation was made in the samples stained by ap2IgY and aTIgY (fig. 37 and 38). In both cases, only small aggregates of *E.coli* cells which were distributed irregularly lit up (representative pictures were taken in two different areas). Also, aTIgY and p2IgY stainings were characterized by a a distinctive signal pattern. The aTIgY staining was spread and halo-shaped, while the ap2IgY signal was intensified in the cell poles. In both cases, the signal coming from IgYs filled the bacteria shape detected by the *E.coli*-specific IgG.

These observations combined with the results of the inhibition assay indicate that ap2IgY and aTIgY might bind specifically with TEM-1. However, it differs from the reference ß-lactamase-IgG1. Several hypotheses are possible to explanation this discrepancy. One of them is that IgYs and ß-lactamase IgG are possibly specific to two different conformations of TEM-1. ATIgY was developed against recombinant TEM-1 precursor protein lacking a 23 amino acids signaling sequence, which is important for transport from the cytoplasm through the membrane into the periplasm. This sequence is present in mature TEM-1. [209] During the translocation of the ß-lactamase from the cytoplasm to the periplasmatic space, the enzyme undergoes protein folding changes necessary to enter the hydrophobic environment of the membrane, and additional changes to exit in the polar, aqueous periplasm to become catalytically active ß-lactamase.[210, 211] It also includes the mechanism of hiding or exposing polar and non-polar residues by ß-lactamase while changing the structural location within the bacteria cell. [204]

Another hypothesis might be a so called phenotypic heterogenicity or bistability of bacteria in one genetically homogeneous population. [212, 213] Both phenomena refer to the changes which randomly occur in some cells of the one population, distinguishing potent cells, what leads to the formation of two subpopulations e.g. expressing the same protein at different levels, or growing with different speed. It usually happens after rapid change of environment or as a response to a treatment. It leads to the assumption that in a given TEM-1-producing *E.coli* population, more potent cells expressing TEM-1 at different level were specifically targeted by ap2IgY and aTIgY during the immunofluorescent staining and neutralized during the inhibition assay. This hypothesis is the most probable, especially, that according to manufacturer, ß-lactamase-IgG1 is specific to the whole class A ß-lactamases and not exclusively to TEM-type. More investigation and supportive evidence is required to confirm this hypothesis, for example by combining immunofluorescent staining of TEM-1 producing *E.coli* using IgYs with TEM-1 mRNA FISH method (described by Rohde et al.) to compare the protein level and transcription among cells. [208]

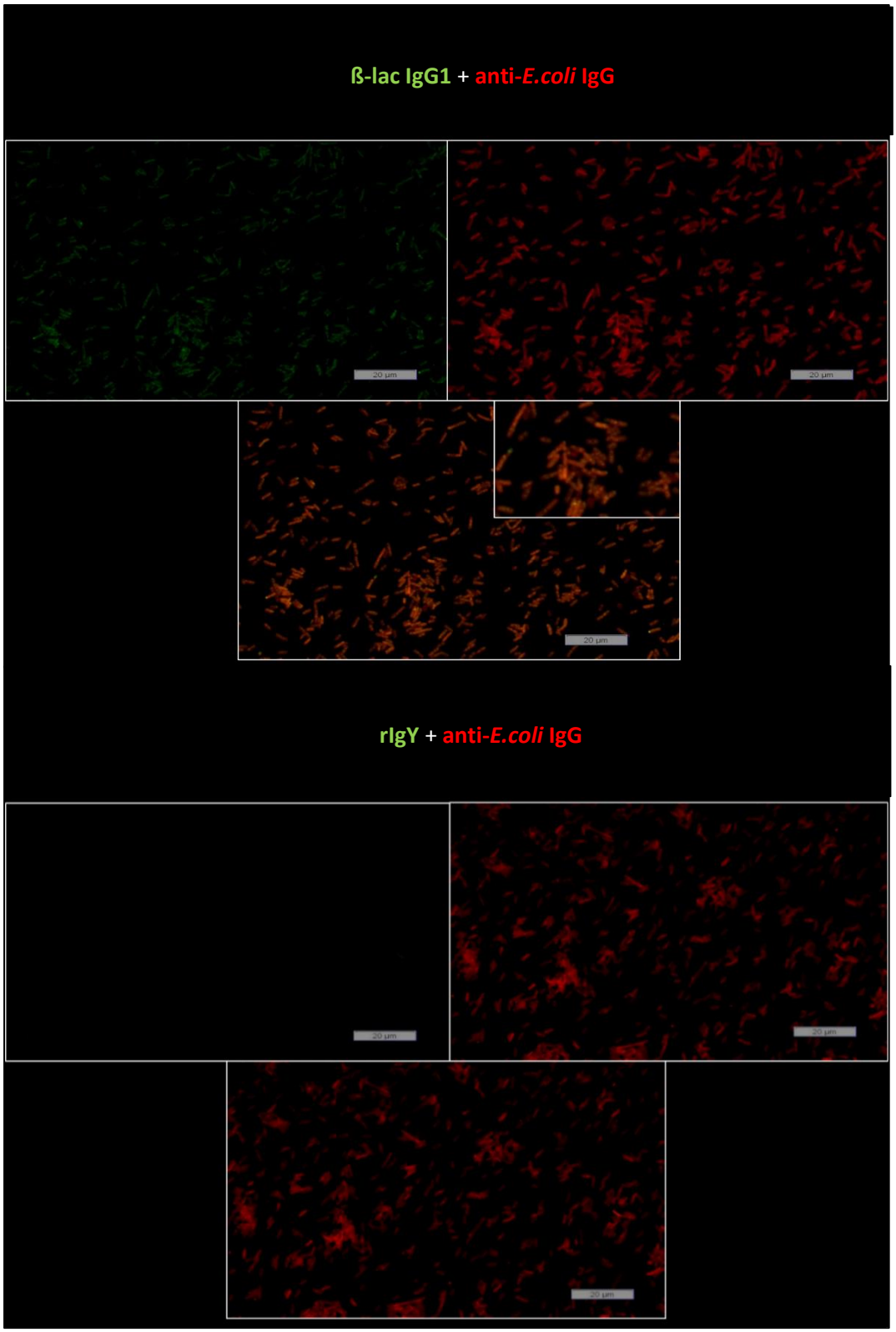


Fig.36. Immunofluorescent staining of TEM-1 in TEM-1-producing *E.coli* culture, positive (upper) and negative (bottom) controls. Before staining, E.coli cells were permeablized with 0.1% Triton x-100. As the next step, *E.coli* were incubated with primary antibodies overnight (ßlac IgG1, anti-*E.coli* IgG, rIgY) and then incubated for 1h with secondary antibodies conjugated with fluorophores for the binding visualization. At the upper picture are: positive control staining with ß-lactamase-IgG1 (green) and *E.coli*-IgG (red), and merge of both. At the bottom picture are: negative control staining with rIgY (did not bind therefore there is no green signal) and *E.coli*-IgG (red), and merge of both.

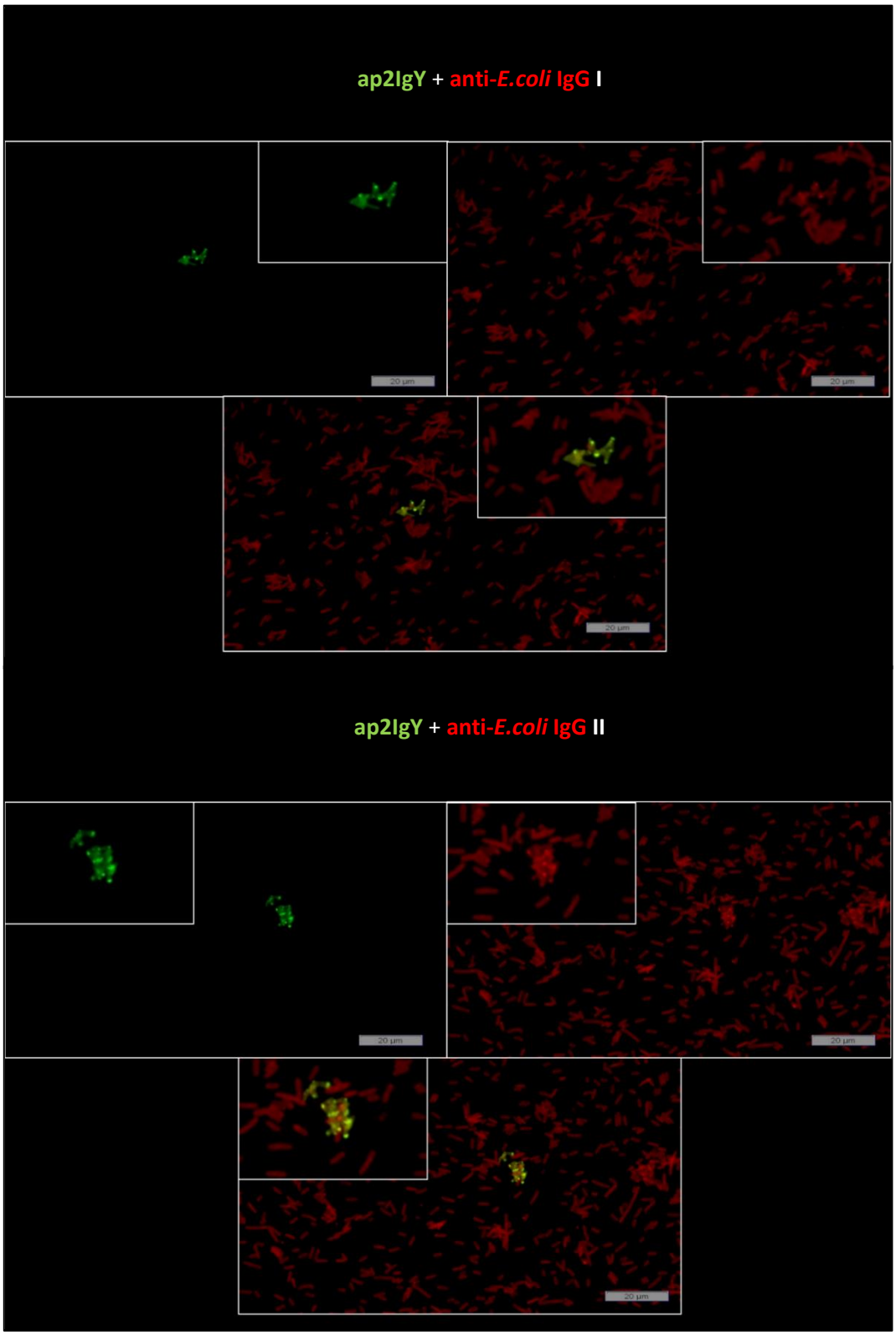


Fig.37. Immunofluorescent staining of TEM-1 in TEM-1-producing *E.coli* culture with ap2IgY, pictures of two different areas are shown: *E.coli*-IgG (red), ap2IgY (green) and merge of both. Before staining, *E.coli* cells were permeablized with 0.1% Triton x-100. As the next step, *E.coli* were incubated with primary antibodies overnight (ap2IgY, anti-E.coli IgG) and then incubated for 1h with secondary antibodies conjugated with fluorophores for the binding visualization.

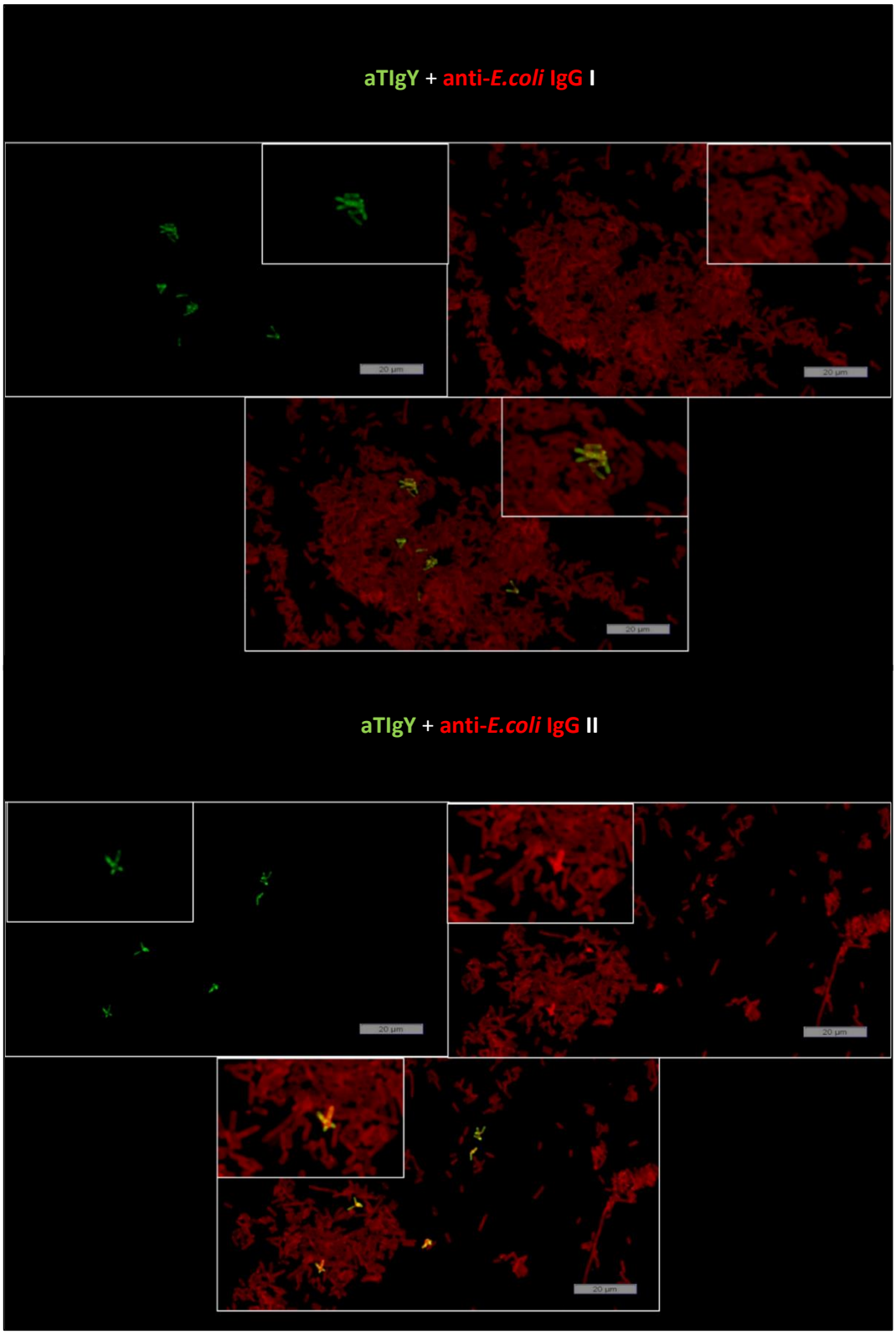


Fig.38. Immunofluorescent staining of TEM-1 in TEM-1-producing *E.coli* culture with aTIgY, pictures of two different areas are shown: E.coli-IgG (red), ap2IgY (green) and merge of both. . Before staining, *E.coli* cells were permeablized with 0.1% Triton x-100. As the next step, *E.coli* were incubated with primary antibodies overnight (aTIgY, anti-E.coli IgG) and then incubated for 1h with secondary antibodies conjugated with fluorophores for the binding visualization.

4.3. IgYs against ß-lactamase TEM-1-producing *E.coli* cells used as alternative to antibiotic treatment reduced the growth of *E.coli in vitro*

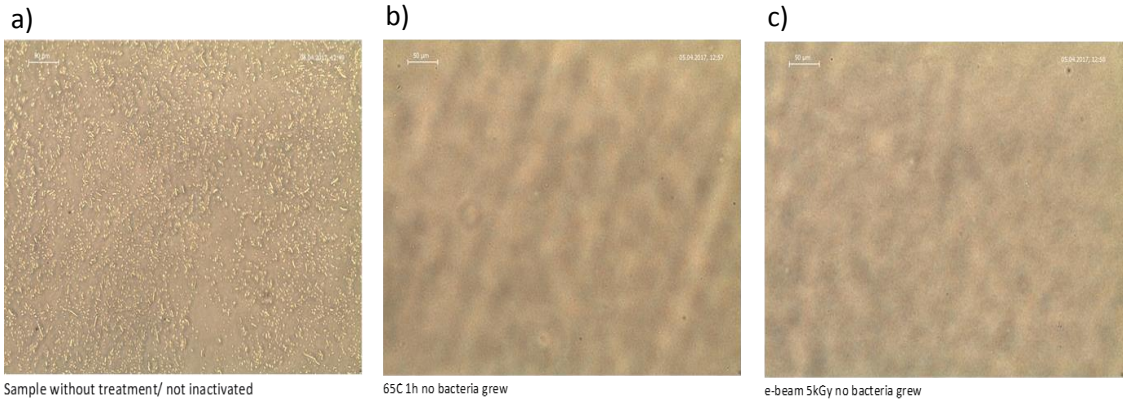
The aim of this strategy was to target and inhibit the growth of TEM-1-producing *E.coli* by specific IgYs developed against their cell surface, without the presence of an antibiotic. There are several studies that reported good results in the treatment against bacteria with specific IgYs obtained after immunizing chickens with inactivated bacterial cells. [162, 202, 214, 215] IgYs produced with this method showed not only bacteria growth inhibitory potential but might also prevent the bacterial colonization by inhibiting their adhesion to epithelium cells. [216]

To obtain highly specific IgYs towards the TEM-1-producing *E.coli* cell surface, two types of *E.coli* inactivation methods for IgY development were assessed. Heat inactivation is a common and effective method of killing bacteria and the temperature used ranges between 60 and 80˚C. The increased temperature cause denaturation of cellular components, such as DNA and ribosomes, and melting of membrane lipids what results in death of bacteria. It might negatively influence the preservation of antigens on the cell surface. [217, 218]

The electron beam irradiation (E-beam) is based on low energy electron irradiation (LEEI) and it is a new method of inactivating pathogens. Since it preserves cell integrity and antigenic structures, it has a big potential in vaccine development. [189] As such, eIgY and hIgY were developed by immunization of chickens with either e-beam-inactivated or heat-treated (65°C) whole cells of TEM-1 producing *E.coli,* respectively. Both types of antibodies were affinity-purified.

This is the first study presenting the use of the E-beam in whole bacterial cell antigens preparation for the development of specific IgYs. After the inactivation of TEM-1-producing *E.coli* by E-beam at 5kGy or incubation at 65˚C for 1h, cells were tested for viability and compared with positive control – TEM-1-producing *E.coli* cultivated in LB. The results are shown in fig.39. Both inactivation methods were successful and no bacteria cell growth was observed, opposite to the positive control.

Fig.39. Viability test of TEM-1-producing *E.coli* heat- and E-beam-inactivated in comparison to non-treated bacteria cells: a) no treatment, b) 65˚C 1h, c) e-beam.



4.3.1. Binding activity of affinity purified IgYs against TEM-1-producing *E.coli*

In order to test the binding activity of the affinity-purified eIgY and hIgY against the TEM-1-producing *E.coli* cell surface, indirect ELISA tests were performed against inactivated *E.coli* cells coated on the plate. The results of ELISA against TEM-1-producing *E.coli* fixed by 4% formaldehyde solution, revealed that eIgY bound better to *E.coli* cells than hIgY and rIgY (used as a negative control)(fig.40). In the two-fold dilution series starting with 80µg/ml as the highest IgY concentration, eIgY showed binding specificity down to the concentration of 1.25µg/ml and significantly differed from ap2IgY and rIgY ($p<0.05$). No significant difference was observed between values obtained with hIgY and rIgY.

These results might indicate that eIgYs more specifically recognized TEM-1-producing *E.coli* cells, because the E-beam indeed preserved the integrity of cells and antigenic epitopes better, unlike the heat-inactivation which more likely disrupted the cell integrity and generated IgYs specific not only to the cell surface but also to the cell content.

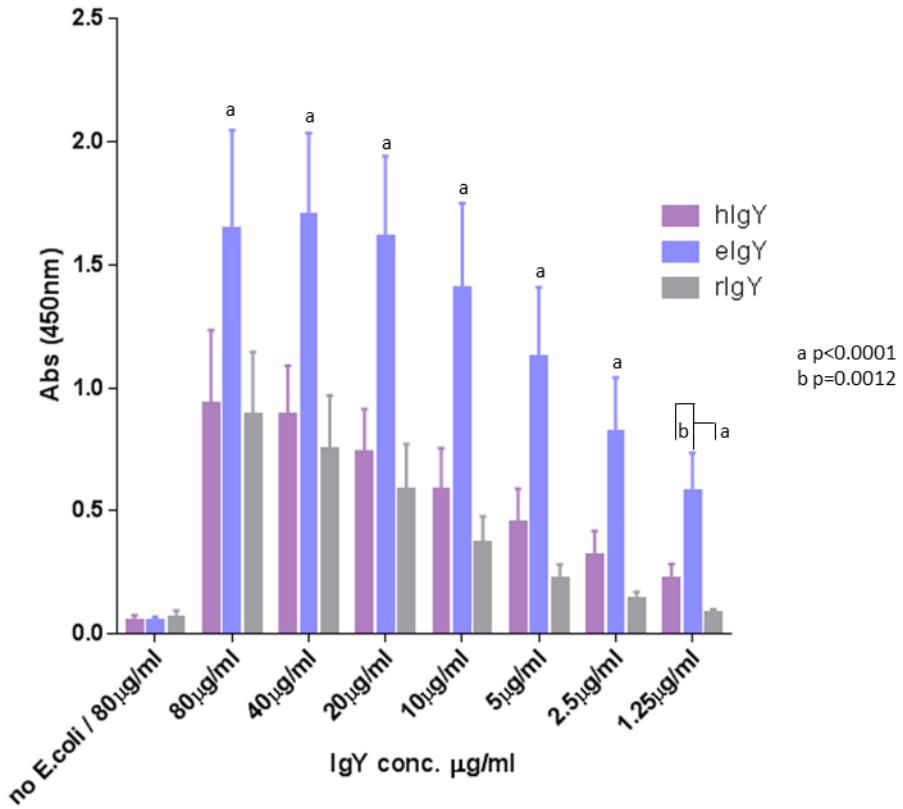


Fig.40. Comparison of binding activity between affinity-purified hIgY and eIgY targeting TEM-1-producing *E.coli* cells. The 96 well plate was coated with the TEM-1-producing *E.coli* cells previously inactivated with 4% formaldehyde solution, in the concentration corresponding to ~$1.5x10^6$ CFU/ml. As the primary antibodies hIgY (affinity-purified IgY against heat-inactivated *E.coli*), eIgY (affinity-purified IgY against e-beam inactivated *E.coli*) and rIgY (non-specific IgY) were used. Secondary anti-IgY antibodies were conjugated to a horse radish peroxidase for the binding visualization. (n=3 performed in triplicates, p-values of less than 0.05 were considered as statistically significant)

4.3.2. In vitro growth inhibition of TEM-1 producing *E.coli* by affinity purified IgYs specific to *E.coli*

To test the influence of affinity-purified IgYs as a compound alternative to antibiotic on the growth of TEM-1-producing *E.coli in vitro*, an inhibition assay was performed, where the TEM-1-prodcuing *E.coli* liquid cell cultures were incubated for 24h with 0.1/ml of either eIgY, hIgY or rIgY, diluted in LB medium without ampicillin. Interestingly, in a contrast to the ELISA results, both eIgY and hIgY inhibited the growth of TEM-1-producing *E.coli in vitro*. As expected, LB and rIgY treatment did not. Surprisingly, the results from OD_{600} measurement clearly did not support that observation: in all of the samples transparency and color changed over the time (fig.41 a).

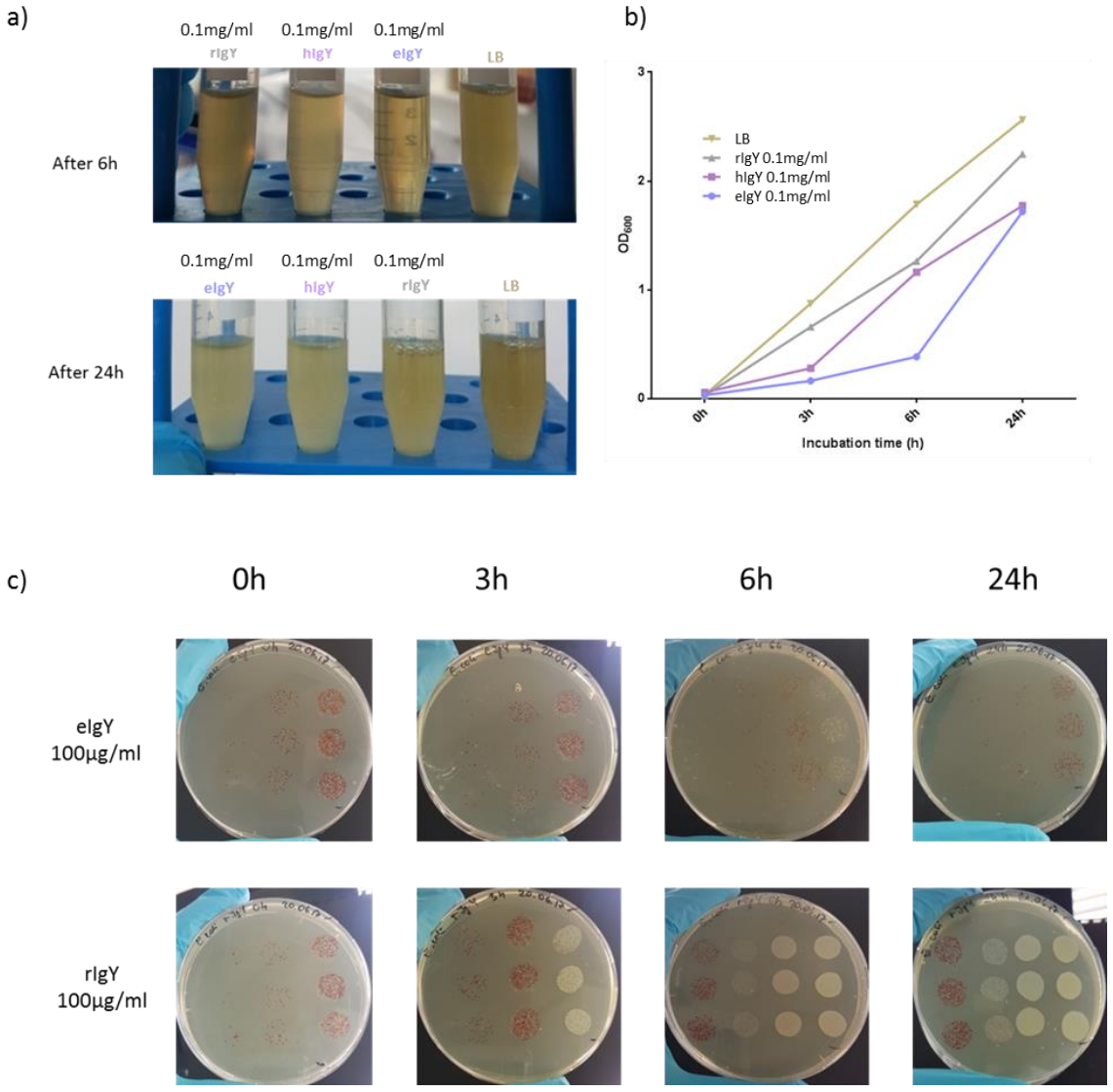


Fig.41. The influence of affinity-purified IgYs targeting TEM-1-producing *E.coli* cells on the growth of the same *E.coli* strain a) The visual changes in samples after 6 and 24h of incubation, b) optical density measurement, (n=1), c) the representative example of drop plates used for the calculation of colony forming unit. *E.coli* inoculates were incubated for 24h in the presence of 100µg/ml eIgY and hIgY. As controls LB medium without ampicillin (no IgY) and non-specific IgYs were used (rIgY). To compare the growth of *E.coli* after incubation with different IgYs by the drop plate method, samples were taken at the time points: 0h, 3h, 6h and 24h.

The result of cell density measurement showed that the *E.coli* density grew for all of the samples (fig.41 b). After 24h, the color in all the samples with IgYs was similar, only LB turned darker, what might be due to metabolites from bacteria cells. Although, more repeats are needed to interpret these data. Fig. 41 c shows a representative example of drop plates established in this inhibition assay. The bacteria cell count revealed that the treatment with eIgY and hIgY significantly decreased the number of CFU/ml in samples treated with TEM-1-producing *E.coli* ($p<0.05$)(fig.42 a). Treatment with rIgY and LB expectedly did not. The growth curves with logarithmic values of TEM-1-producing *E.coli* showed a steady declination 6h after a treatment with eIgY or hIgY. Both were significantly different from those after treatment with rIgY and LB ($p<0.05$)(fig.42 b).

These results might indicate that specific and affinity purified eIgY and hIgY developed against the cell surface of TEM-1-producing *E.coli* show the inhibitory effect on the TEM-1-producing *E.coli in vitro* at concentrations as low as 0.1mg/ml. These observations are comparable to a study of Sunwoo et al., where specific IgYs were developed against ETEC 987P *E.coli.* [214] Although these, were not based on the inactivated whole bacteria cell antigen but on the cell lysate. Compared to the current study, they used a similar IgY concentration for their inhibition test: 0.09mg/ml (in here 0.1mg/ml) but slightly different concertation of *E.coli* cells ~ 10^5 CFU/ml (in here ~ $2x10^6$ CFU/ml). The mechanism of the growth inhibition of bacteria by eIgY and hTIgY was not explored. However, it might be that by targeting and adhering to the surface of *E.coli*, they blocked biological functions of bacteria, overlapped surface virulence factors and blocked the signaling between cells, what could result in the suppression of growth and multiplication.

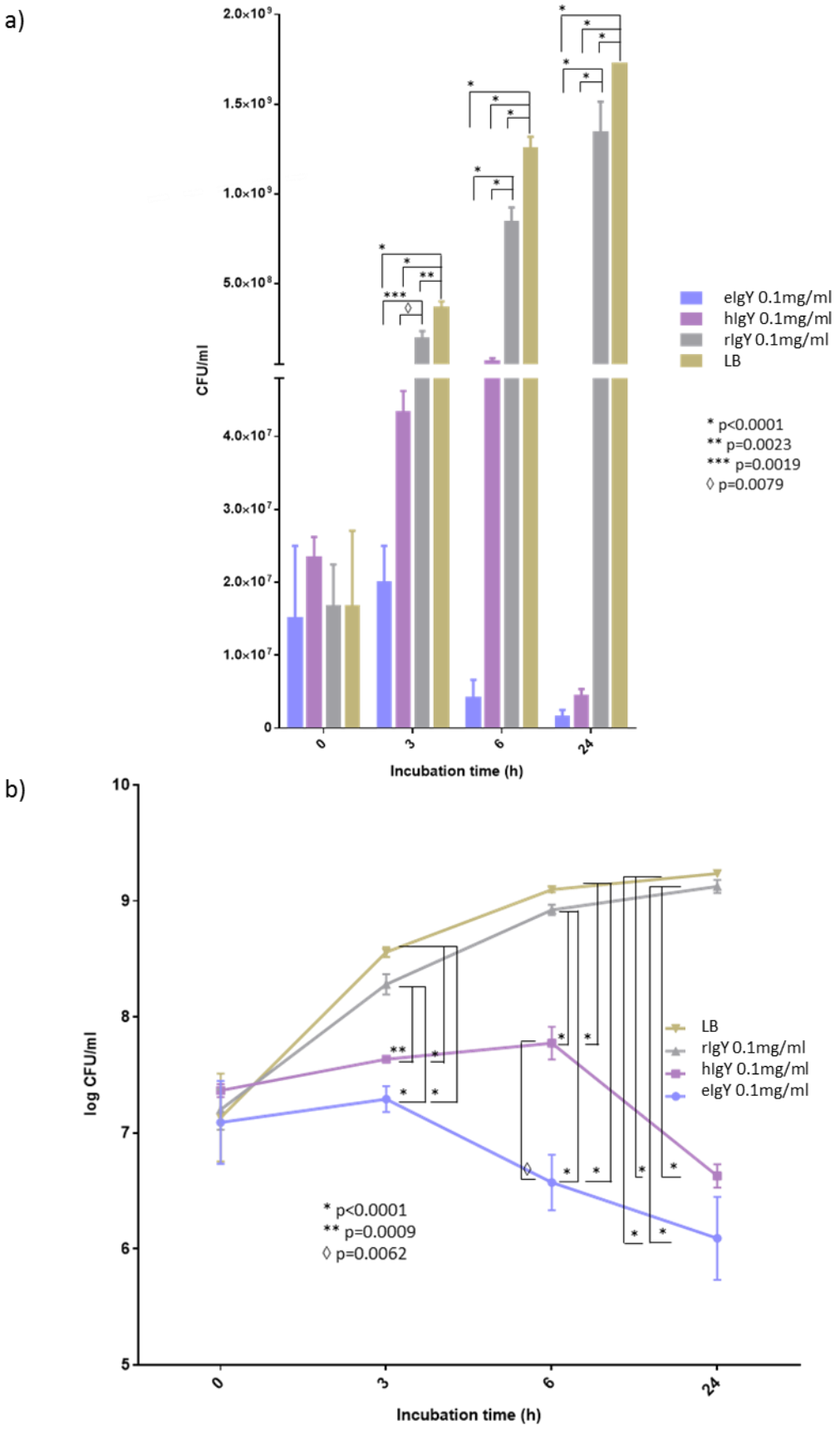


Fig.42. Inhibitory effect of affinity-purified IgYs targeting TEM-1-producing *E.coli* cells on TEM-1-producing *E.coli*: a) influence on number of colony forming units, b) influence on growth curve. (n=3, p-values of less than 0.05 were considered as statistically significant)

4.3.3. Immunofluorescent staining of TEM-1-producing *E.coli* using affinity-purified IgYs developed against TEM-1-producing *E.coli* cells

To visualize the binding activity of eIgY and hIgY to the cell surface of TEM-1-producing *E.coli* the immunofluorescence staining was employed (fig.43-45). As a positive control, the *E.coli*-specific monoclonal IgG was used, and as a negative control, a rIgY. DNA was stained with DAPI. The immunofluorescence staining was done in two ways: primary antibodies were either incubated for 1h or overnight. The difference in signal intensity was not noticed between the incubation times, but due to technical problems amount of bacteria varies among samples.

Both eIgY and hIgY showed specific binding to cell surface of TEM-1-producing *E.coli*, the signal was strong and clearly overlapping with the staining pattern given by the positive control (fig. 44 & 45). Furthermore, the signal coming from a cell surface targeted by hIgY or eIgY, surrounded the DAPI signal inside the cell nicely. Importantly, rIgY did not give any signal meaning there was no unspecific binding (fig. 43). Additionally, subpopulations in TEM-1-producing *E.coli* were noticed on the fig. 43 and 45 (white arrows), including the positive-control staining. It might have appeared due to phenotypic heterogenicity. [211, 212] According to this theory, within the one genetically homogeneous population of bacteria some phenotypic changes can occur. It may happen as a result of the stressful environmental conditions (e.g. presence of antibiotics, temperature changes or pH changes) or spontaneously. They can refer to many attributes and characteristics of bacteria, including e.g. enzymes, toxins, virulent factors expressed at the different level or it may appear as a change of growth speed among cells. It may result in the formation of two subpopulations, which might give later a different staining pattern within the same sample.

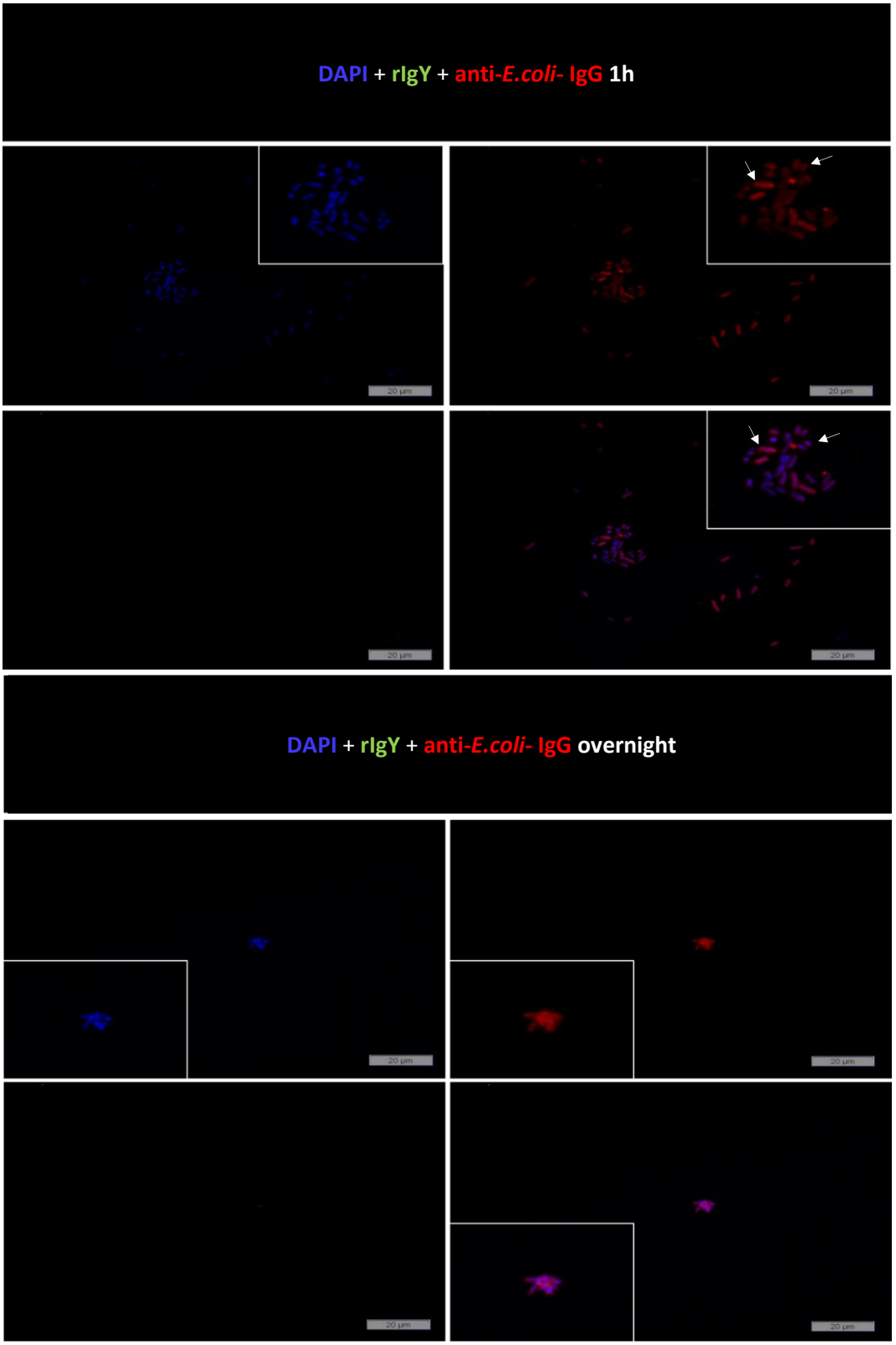


Fig.43. Immunofluorescent staining of TEM-1-producing *E.coli* cells, after 1h incubation (upper) and overnight incubation (bottom) with primary antibodies. Cultivated and harvested *E.coli* were first incubated with primary antibodies rIgY, anti-*E.coli* IgG (1h/overnight) and then incubated for 1h with the secondary antibodies conjugated with fluorophores for the binding visualization. A positive control staining with *E.coli*-IgG (red), DAPI (blue), negative control staining with rIgY (no green signal detected) and merge of three. White arrows show cells subpopulations.

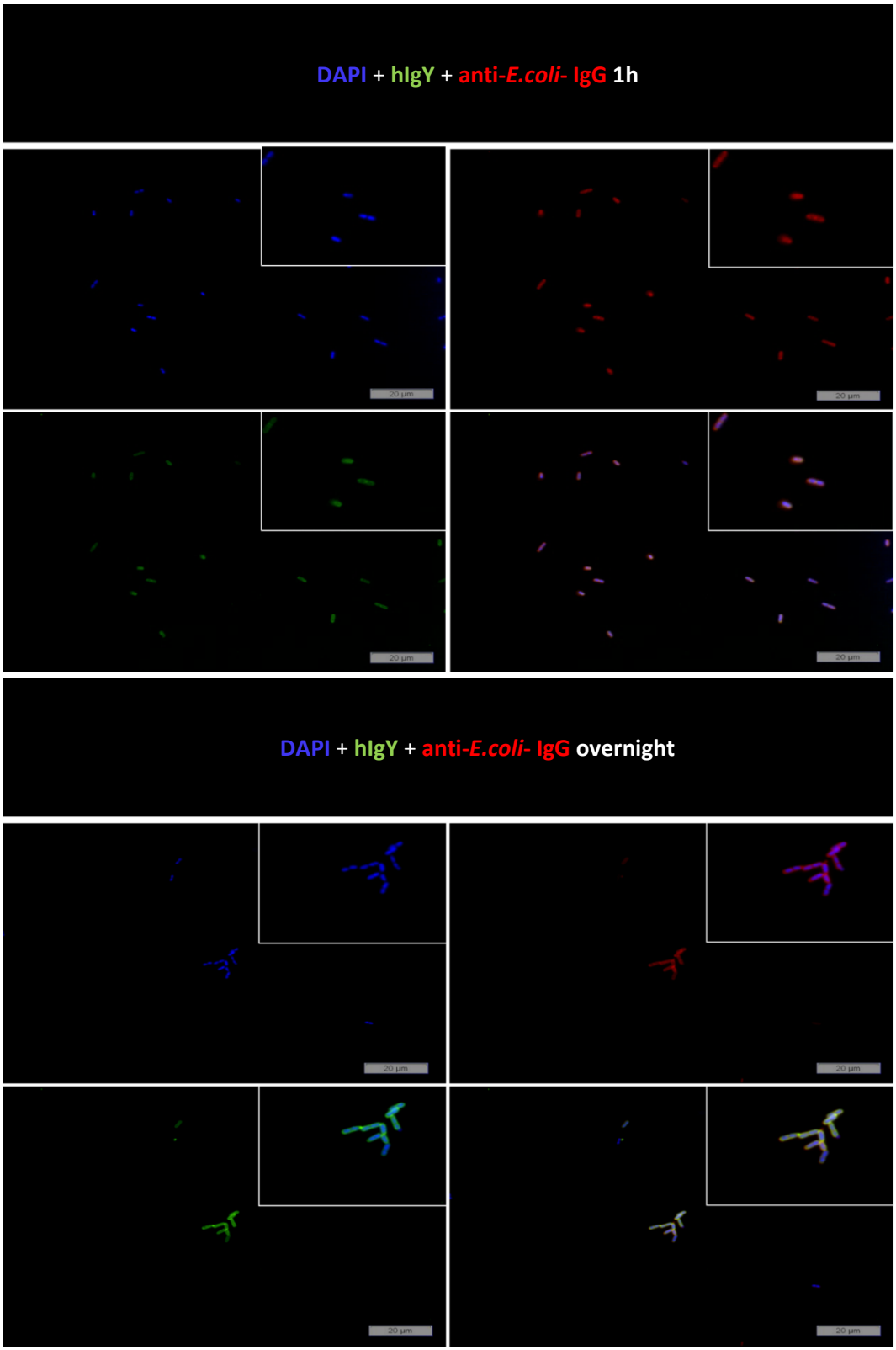


Fig.44. Immunofluorescent staining of TEM-1-producing *E.coli* cells, after 1h incubation (upper) and overnight incubation (bottom) with primary antibodies. Cultivated and harvested *E.coli* were first incubated with primary antibodies hIgY, anti-E.coli IgG (1h/overnight) and then incubated for 1h with the secondary antibodies conjugated with fluorophores for the binding visualization.A positive control staining with *E.coli*-IgG (red), DAPI (blue), staining with hIgY (green) and merge of three.

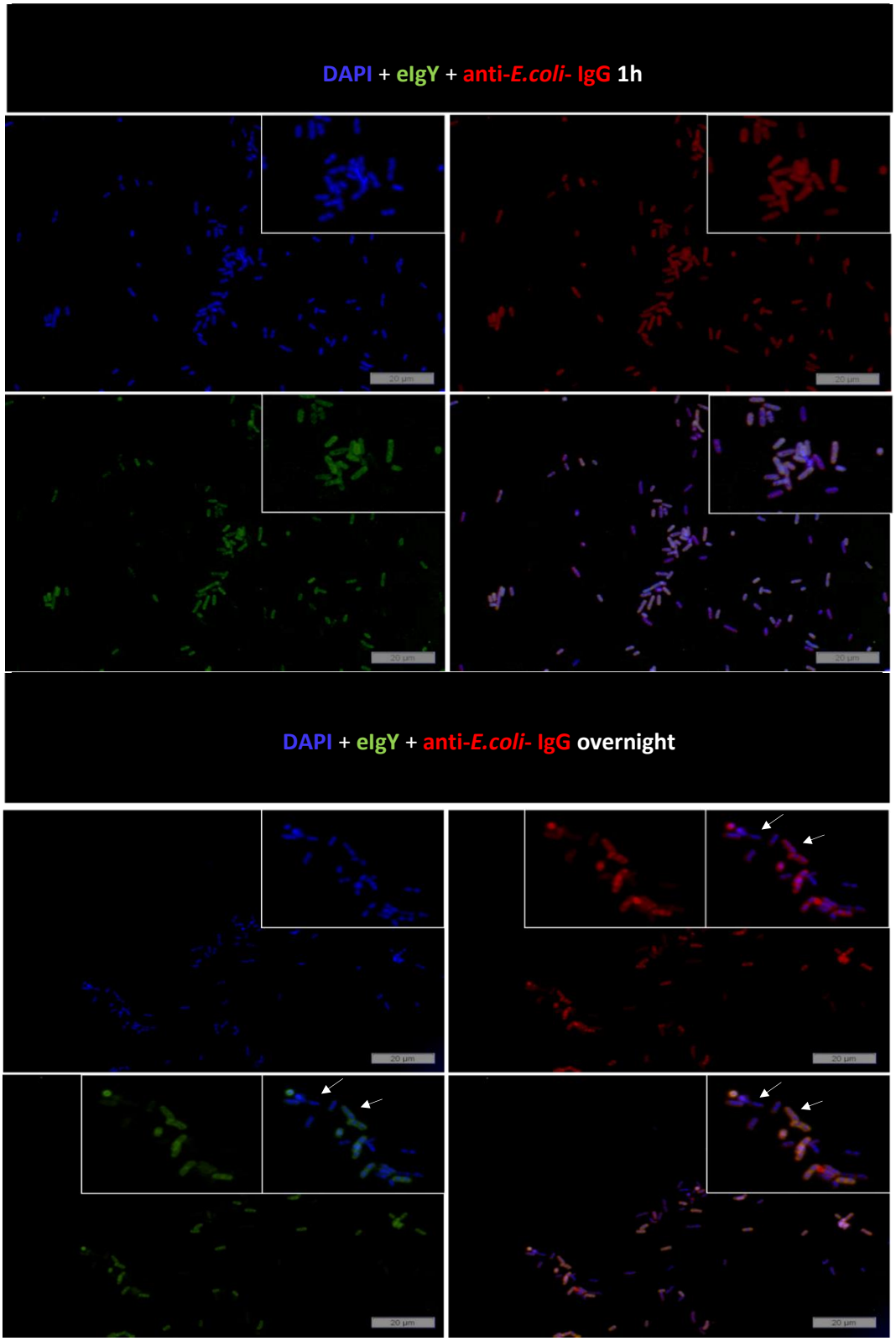


Fig.45. Immunofluorescent staining of TEM-1-producing *E.coli* cells, after 1h incubation (upper) and overnight incubation (bottom) with primary antibodies. Cultivated and harvested *E.coli* were first incubated with primary antibodies eIgY, anti-E.coli IgG (1h/overnight) and then incubated for 1h with the secondary antibodies conjugated with fluorophores for the binding visualization.A positive control staining with *E.coli*-IgG (red), DAPI (blue), staining with eIgY (green) and merge of three. White arrows show cells subpopulations.

Conclusions and perspectives

5. Conclusions and perspectives

This study presents a model system to develop specific IgYs against a therapeutic target of interest. Specifically, different types of IgYs were generated against ß-lactamase TEM-1-producing *E.coli* (BW25113 *ΔbamBΔtolC*)as a representative target and had the typical configuration of avian antibodies, were highly specific to their antigens and inhibited the growth of ß-lactamase TEM-1-producing *E.coli* (BW25113 *ΔbamBΔtolC*) *in vitro*. Two strategies were design to target the TEM-1-producing *E.coli* with IgYs: as a complement to antibiotics (IgYs against the enzyme TEM-1 used in combination with ampicillin) and as an alternative to antibiotics (IgYs against the bacteria TEM-1-producing *E.coli* without the addition of ampicillin).

In the first strategy, IgYs were developed against either the whole TEM-1 (TIgY and aTIgY) or only the active site of TEM-1 (p1IgY, p2IgY, ap1IgY and ap2IgY). For this, chickens were immunized with Beta lactamase TEM precursor protein and *in silico* designed short peptides mimicking the active site, respectively. Among 7 identified catalytic and conservative residues for TEM-group ß-lactamases, 2 pairs of residues were chosen as a base for 2 11-amino-acid peptides synthesised to mimic the active site of TEM-1: Ser70 and Lys73 (Sequence 1 RFPMMSTFKVL, located on a H2 helix), and Glu166 and Asn170 (Sequence 2 TRLDRWEPELN, located on the Ω-loop). The ELISA tests revealed that among the precipitation-purified short peptides based IgYs, only p2IgY had a binding activity towards the active site of Beta lactamase TEM precursor protein. Moreover, TIgY showed the highest binding activity towards Beta lactamase TEM precursor protein among all precipitation-purified IgYs. Although, in the ELISA tests with TEM-1-producing *E.coli* lysate only TIgY and affinity-purified aTIgY showed binding activity compared to the other IgYs in their respective groups. Interestingly, not only TIgY (against TEM-1), but also p2IgY (against sequence 2) showed an inhibitory effect on the growth of TEM-1-producing *E.coli in vitro* in the presence of ampicillin. Similar results were obtained with their affinity-purified counterparts (aTIgY and ap2IgY), however, using only a fraction of the concentration (5 mg/mL vs 0.1 mg/mL). Their reactivity towards TEM-1-producing *E.coli* was confirmed by immunofluorescence. It might be that ap2IgY and aTIgY inactivate the TEM-1 by targeting the active site and building an antibody coat around the enzyme, respectively, and thereby preserve the antimicrobial activity of ampicillin. These hypotheses though, require further investigation. Nevertheless, these results indicate that the Ω-loop is potentially a good target for IgYs against TEM-1.

In the second strategy, IgYs were developed against TEM-1-producing *E.coli*. In this case, chickens were immunized with *E.coli* cells inactivated by one of the two methods: e-beam and heat

treatment. Although only eIgY showed binding activity compared to controls using in ELISA tests, both affinity-purified eIgY (e-beam) and hIgY (heat treatment) showed reactivity towards TEM-1-producing *E.coli* in the immunofluorescence assay and inhibited the bacteria's growth *in vitro* without the presence of ampicillin. The observation could be explained by the possibility that IgYs developed against the bacteria surface, target the cells by building an antibody coat around them and, therefore, inhibit their biological functions.

It is of interest to further determine the specificity of ap2IgY and aTIgY and confirm the presence of bacterial subpopulations with additional experiments. For instance, it would be beneficiary to explore the correlation between the protein detected by the IgYs and the gene expression pattern of TEM-1 by immunofluorescence and mRNA FISH, respectively.[208]

Additionally, the stability and mutability of the TEM-1 gene construct in *E.coli* BW25113 *ΔbamBΔtolC* should be tested and compared among the subpopulations observed in the immunofluorescence experiments, e.g. using the next generation sequencing. For this and other analysis, the subpopulation could be separated by fluorescence-activated cell sorting. Furthermore, the specificity of interaction between ap2IgY/aTIgY and TEM-1 could be tested by an *in vitro* inhibitory assay with non-ß-lactamase-producing *E.coli* strain and strains producing different class of ß-lactamase. Also, a control inhibition assay without the presence of ampicillin could be performed. Subsequently, it would be advantageous to have an insight into a molecular interaction between IgYs and TEM-1. This could be done *via* binding affinity and kinetic tests using e.g. Plasmon Surface Resonance. Though, the screening assay should be design in the way to consider big molecular differences between ligand and analyte components (TEM-1 ~30kDa, IgY 180 kDa).

The cross-reactivity of hIgY and eIgY could be investigated by an inhibition assay with different bacteria species than *E.coli* and with samples where the *E.coli* BW25113 *ΔbamBΔtolC* would be mixed with other *E.coli* strains. It would also bring more insight into the effectivity of an antigen preservation of the heat and the e-beam methods used to inactivate the bacteria for chicken immunization and IgY production. To further explore the activity of these IgYs and their influence on the *E.coli* physiology, a bacterial flow cytometry can be performed using these and a panel of other markers. This method has many microbiological applications. [219]

IgYs developed in this study might also be good candidates for further investigation as a broad-spectrum treatment against a variety of ESBL-producing *E.coli*. The aTIgY which was developed against TEM-1 might also target its derivatives, as they have similar 3D structure with single amino acids mutations in the sequence. The ap2IgY was generated against catalytic and conservative residues, characteristic for the whole class A of ß-lactamases. It would be interesting to test these IgYs *in vitro* against patient's *E.coli* isolates with TEM-ESBL profile. IgYs based on the heat and the e-beam inactivated bacteria could be newly developed against these isolates and tested.

To confirm the good inhibitory effect of (a)TIgY , (a)p2IgY, eIgY and hIgY on TEM-1-producing *E.coli* that was observed in-vitro, they should be further investigated in an animal infectious model to test their activity complementary or alternatively to antibiotics *in vivo*. Several studies reported a positive effect of orally introduced specific IgYs against different bacteria in animal models, e.g. against *C.difficile* in hamster, *H.pylori* in mouse and *E.coli* in piglets.[140, 220–222] The ultimate intention of this research is to develop an effective and save IgY-based oral therapy for human patients against ESBL-producing gram negative bacteria. Human studies on specific IgYs delivered to patients orally showed a good effect against bacterial infections such as *H.pylori* or *P.aeruginosa.*[148, 154]

As an egg's component, IgYs are consider as save and non-toxic. An allergy to eggs can be overtaken by affinity purification, separating IgYs from other proteins. Additionally, immunotherapy does not cause bacterial resistance. Thereafter, in this context, two important parameters should be first studied: stability of the developed IgYs and absence of a gut microbiome shift after the treatment (to not disturb a gut's flora balance). As such, in an animal study, except an infection rate analysis after IgY treatment, a metabolic, stool tests, and next generation sequencing to monitor the gut flora change could be performed.

An oral IgY therapy against ESBL-producing gram negative gastrointestinal bacteria would be of specific interest. It is that asymptomatic infection can spread to other localizations, such as lungs, urinary tract, blood system. Usually, these infections are already not responsive to standard antibiotics due to the previous antibiotic treatment and additionally they can be easily transmitted to other patients in hospitals. It is especially dangerous to chronically ill patients. [90, 223–225]

Only one clinical study on IgYs treatment against ESBL-producing *E.coli* could be found, where the IgYs were developed against freeze-dried whole *E.coli* cells and administrated to hospital patients. [226] Unfortunately, due to the problems with the study design it could not be completed. Nevertheless, the study confirmed that the treatment with IgYs exhibited no toxic effects on patients; side effects which appeared were considered as mild. It also confirms a huge niche in this research field and an importance of developing IgYs against gastrointestinal ESBL-producing *Enterobacteriaceae* as a prophylaxis and a solution for decreasing effectivity of the antibiotic therapy.

Summary

6. Summary

Zusammenfassung der Arbeit

Dissertation zur Erlangung des akademischen Grades

Dr. rer. med.

An der Medizinischen Fakultät der Universität Leipzig

Titel

IgY antibodies against bacterial infection

Development of candidate IgY antibodies against ESBL-producing gram-negative bacteria for oral therapy

Eingereicht von

M. Sc. Julia Dominika Zając

Angefertig an/in

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Fraunhofer Institute for Immunology and Cell Therapy, Leipzig

Betreut von

Prof. Dr. Friedemann Horn

The antibiotic resistance is developing faster than the development of new antibiotics, what increases the need of finding efficient alternative therapies. On the 27th of February 2017, WHO published the 'Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics' in Geneva, Switzerland. This document focuses on 12 antibiotic-resistant bacteria, and among them the Extended Spectrum Beta-Lactamase (ESBL)-producing *Enterobacteriaceae* family (including *E.coli*) is classified to a group of the critical priority.

The IgY technology (based on chicken polyclonal immunoglobulins Y – IgY) brings a lot of advantages in the fight against resistant bacteria including: a) fast and effective production of highly specific antibodies, b) reduction of risk of bacterial resistance to a therapy, c) do not activate the complement, do not bind to A and G proteins, mammalian antibodies like rheumatoid factors or human anti-murine antibodies, and the cell surface Fc receptor, d) are characterized by high stability, high avidity and low cross-reactivity.

The general idea of this study was to develop candidate specific IgY antibodies for an oral therapy targeting the ESBL-producing gram negative bacteria. As the family of ESBLs constantly grows and there is lack of their clear classification in the literature, the specific aim was to build a proof of concept study based on the parental enzyme ß-lactamase TEM-1 to investigate different specific IgYs strategies to inhibit the growth of TEM-1 producing *E.coli*.

This research included a bioinformatic analysis of the TEM-1 structure in the context of TEM-derivative ESBLs. Then, two IgY strategies were designed to target the ß-lactamase TEM-1-producing *E.coli* (BW25113 *ΔbamBΔtolC*) with IgYs: as a complement to antibiotics (IgYs against the enzyme TEM-1 used in combination with ampicillin) and as an alternative to antibiotics (IgYs against the bacteria TEM-1-producing *E.coli* without the addition of ampicillin). A good inhibitory effect of (a)TIgY, (a)p2IgY (in the presence of ampicillin) and eIgY, hIgY (without the ampicillin) on TEM-1-producing *E.coli* was observed *in vitro*. Moreover, they had the typical configuration of avian antibodies and were highly specific to their antigens.

This study presents a model system to develop specific IgYs against a therapeutic target of interest. The activity of these IgYs complementary or alternatively to antibiotics should be further investigated *in vivo* in an animal infectious model. IgYs developed in this study might also be good candidates for further investigation as a broad-spectrum treatment against a variety of ESBL-producing *E.coli*. The aTIgY which was developed against the whole TEM-1 might also target its derivatives, as they have similar 3D structure with single amino acids mutations in the sequence. The ap2IgY was generated against catalytic and conservative residues, characteristic for the whole class A of ß-lactamases, thus it might target also the active site of ESBL-s from this class. The strategy used to generate eIgY and hIgY was efficient and IgYs could be generated directly against ESBL-producing bacteria.

Authorship declaration and confirmation

7. Authorship declaration and confirmation

Erklärung über die eigenständige Abfassung der Arbeit

Hiermit erkläre ich, dass ich die vorliegende Arbeit (IgY antibodies against bacterial infection) selbstständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar eine Vergütung oder geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren. Die aktuellen gesetzlichen Vorgaben in Bezug auf die Zulassung der klinischen Studien, die Bestimmungen des Tierschutzgesetzes, die Bestimmungen des Gentechnikgesetzes und die allgemeinen Datenschutzbestimmungen wurden eingehalten. Ich versichere, dass ich die Regelungen der Satzung der Universität Leipzig zur Sicherung guter wissenschaftlicher Praxis kenne und eingehalten habe.

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